

IAP4 Rec'd PCT/PTO 12 DEC 2005

PTO-1390 (Rev. 07-2005)

Approved for use through 3/31/2007. OMB 0651-0021

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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A SUBMISSION UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER 26788-024 U.S. APPLICATION NO. (If known) 107560377 37 CFR 1.5) TBD
INTERNATIONAL APPLICATION NO. PCT/US2004/019229	INTERNATIONAL FILING DATE 10 June 2004	PRIORITY DATE CLAIMED 12 June 2003
TITLE OF INVENTION CONSERVED HBV AND HCV SEQUENCES USEFUL FOR GENE SILENCING		
APPLICANT(S) FOR DO/EO/US Nucleonics Inc.		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a submission under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a submission under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input checked="" type="checkbox"/> The US has been elected (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ul style="list-style-type: none"> a. <input checked="" type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). </p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). <ul style="list-style-type: none"> a. <input type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). </p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ul style="list-style-type: none"> a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. </p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>		
<p>Items 11 to 20 below concern document(s) or information included:</p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input type="checkbox"/> A preliminary amendment.</p> <p>14. <input checked="" type="checkbox"/> An Application Data Sheet under 37 CFR 1.76.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A power of attorney and/or change of address letter.</p> <p>17. <input checked="" type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13(er.2 and 37 CFR 1.821- 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published International Application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p>		

This collection of information is required by 37 CFR 1.414 and 1.491-1.492. The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 15 minutes to complete, including gathering information, preparing, and submitting the completed form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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U.S. APPLICATION NO. (if known)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER																																																																																																																												
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<p>The following fees have been submitted</p> <table border="1"> <tr> <td>21. <input checked="" type="checkbox"/> Basic national fee (37 CFR 1.492(a)).....</td> <td>\$300</td> <td>CALCULATIONS</td> <td>PTO USE ONLY</td> </tr> <tr> <td></td> <td>\$ 300.00</td> <td></td> <td></td> </tr> <tr> <td>22. <input checked="" type="checkbox"/> Examination fee (37 CFR 1.492(c))</td> <td></td> <td></td> <td></td> </tr> <tr> <td>If the written opinion prepared by ISA/US or the International preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4).</td> <td>\$0</td> <td>\$</td> <td></td> </tr> <tr> <td>All other situations.....</td> <td>\$200</td> <td>200.00</td> <td></td> </tr> <tr> <td>23. <input checked="" type="checkbox"/> Search fee (37 CFR 1.492(b))</td> <td></td> <td></td> <td></td> </tr> <tr> <td>If the written opinion of the ISA/US or the International preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4).</td> <td>\$0</td> <td>\$</td> <td></td> </tr> <tr> <td>Search fee (37 CFR 1.443(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority.....</td> <td>\$100</td> <td>400.00</td> <td></td> </tr> <tr> <td>International Search Report prepared by an ISA other than the US and provided to the Office or previously communicated to the US by the IS.....</td> <td>\$400</td> <td></td> <td></td> </tr> <tr> <td>All other situations.....</td> <td>\$300</td> <td></td> <td></td> </tr> <tr> <td colspan="2">TOTAL OF 21, 22 and 23 =</td> <td>900.00</td> <td></td> </tr> <tr> <td colspan="4"> <input checked="" type="checkbox"/> Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing in compliance with 37 CFR 1.821(c) or (e) or computer program listing in an electronic medium) (37 CFR 1.492(j)). The fee is \$250 for each additional 50 sheets of paper or fraction thereof. </td> </tr> <tr> <td>Total Sheets</td> <td>Extra Sheets</td> <td>Number of each additional 50 or fraction thereof (round up to a whole number)</td> <td>RATE</td> </tr> <tr> <td>150 - 100 =</td> <td>50 /50 =</td> <td>x \$250</td> <td>\$ 250.00</td> </tr> <tr> <td colspan="4">Surcharge of \$130.00 for furnishing any of the search fee, examination fee, or the oath or declaration after the date of commencement of the national stage (37 CFR 1.492(h)).</td> </tr> <tr> <td>CLAIMS</td> <td>NUMBER FILED</td> <td>NUMBER EXTRA</td> <td>RATE</td> </tr> <tr> <td>Total claims</td> <td>66 - 20 =</td> <td>46</td> <td>x \$ 50</td> <td>\$ 2,300.00</td> </tr> <tr> <td>Independent claims</td> <td>16 - 3 =</td> <td>13</td> <td>x \$200</td> <td>\$ 2,800.00</td> </tr> <tr> <td colspan="3">MULTIPLE DEPENDENT CLAIM(S) (If applicable)</td> <td>+</td> <td>\$360</td> </tr> <tr> <td colspan="3"></td> <td>TOTAL OF ABOVE CALCULATIONS =</td> <td>\$ 6,180.00</td> </tr> <tr> <td colspan="4"> <input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. Fees above are reduced by %. </td> <td></td> </tr> <tr> <td colspan="4"></td> <td>SUBTOTAL = \$ 6,180.00</td> </tr> <tr> <td colspan="4">Processing fee of \$130.00 for furnishing the English translation later than 30 months from the earliest claimed priority date (37 CFR 1.492(l)).</td> <td></td> </tr> <tr> <td colspan="4"></td> <td>TOTAL NATIONAL FEE = \$ 6,180.00</td> </tr> <tr> <td colspan="4">Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property</td> <td></td> </tr> <tr> <td colspan="4"></td> <td>TOTAL FEES ENCLOSED = \$ 6,180.00</td> </tr> <tr> <td colspan="4"></td> <td>Amount to be refunded: \$</td> </tr> <tr> <td colspan="4"></td> <td>Amount to be charged \$ 6,180.00</td> </tr> </table>			21. <input checked="" type="checkbox"/> Basic national fee (37 CFR 1.492(a)).....	\$300	CALCULATIONS	PTO USE ONLY		\$ 300.00			22. <input checked="" type="checkbox"/> Examination fee (37 CFR 1.492(c))				If the written opinion prepared by ISA/US or the International preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4).	\$0	\$		All other situations.....	\$200	200.00		23. <input checked="" type="checkbox"/> Search fee (37 CFR 1.492(b))				If the written opinion of the ISA/US or the International preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4).	\$0	\$		Search fee (37 CFR 1.443(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority.....	\$100	400.00		International Search Report prepared by an ISA other than the US and provided to the Office or previously communicated to the US by the IS.....	\$400			All other situations.....	\$300			TOTAL OF 21, 22 and 23 =		900.00		<input checked="" type="checkbox"/> Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing in compliance with 37 CFR 1.821(c) or (e) or computer program listing in an electronic medium) (37 CFR 1.492(j)). 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- d. Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the International Application to pending status.

SEND ALL CORRESPONDENCE TO:

Potter Anderson & Corroon LLP
1313 N. Market Street
Hercules Plaza
Wilmington, DE 19801



SIGNATURE
Jeffrey B. Safran

NAME

54,889

REGISTRATION NUMBER

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Application No.: TBD

Filing Date: December 12, 2005

First Named Inventor: Catherine J. Pachuk

Title: Conserved HBV and HCV Sequences Useful for Gene Silencing

Group Art Unit: TBD

Examiner: TBD

Attorney Docket No.: 26788-024

- Transmittal Letter to the US Designated/Elected Office (DO/EO/UD) Concerning a Submission Under 35 USC 371
- Application Data Sheet
- Copy of the International Application as Filed
- Specification (133 pages, 15 drawings)
- Sequence Listing (Hard Copy and Diskette)
- Receipt Card

APPLICATION DATA SHEET**Inventor Information****IAP9 Rec'd PCT/PTO 12 DEC 2009****Inventor One**

Given Name: Catherine J.
 Family Name: Pachuk
 Postal Address Line One: 3212 New Park Circle
 City: Lansdale
 State or Province: Pennsylvania
 Country: U.S.A.
 Postal or Zip Code: 19446
 Citizenship Country: U.S.A.

Inventor Two

Given Name: Chandrasekhar
 Family Name: Satishchandran
 Postal Address Line One: 605 Shepard Lane
 City: Lansdale
 State or Province: Pennsylvania
 Country: U.S.A.
 Postal or Zip Code: 19446
 Citizenship Country: U.S.A.

Inventor Three

Given Name: Vincent R.
 Family Name: Zurawski Jr.
 Postal Address Line One: 1269 North Gate Road
 City: West Chester
 State or Province: Pennsylvania
 Country: U.S.A.
 Postal or Zip Code: 19382
 Citizenship Country: U.S.A.

Inventor Four

Given Name: Liat
 Family Name: Mintz
 Postal Address Line One: 2 Kulessa Court
 City: East Brunswick
 State or Province: New Jersey
 Country: U.S.A.
 Postal or Zip Code: 08816
 Citizenship Country: Israel

Correspondence Information

Customer No.: 26691

Application Information

Title Line One: CONSERVED HBV AND HCV
SEQUENCES USEFUL FOR GENE
SILENCING

Int'l Application Number: PCT/US2004/019229
Int'l Filing Date: 10 June 2004
Total Drawing Sheets: 17
Application Type: Utility
Docket Number: 26788-024

Priority Information

This application is a 371 of PCT Application No. PCT/US/2004/019229, filed June 10, 2004, now pending, which claims priority from U.S. Provisional Application No. 60/478,076, filed June 12, 2003, each of which is incorporated by reference herein in their entireties. This application also claims priority from U.S. Provisional Application No. 60/638,294, filed December 22, 2004, which is incorporated by reference herein in its entirety.

Representative Information

Registration Number One: 54,689 Jeffrey B. Safran

TITLECONSERVED HBV AND HCV SEQUENCES USEFUL
FOR GENE SILENCING5 CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application Serial No. 60/478,076 filed on June 12, 2003, which is hereby incorporated by reference in its entirety.

10 FIELD OF THE INVENTION

This invention relates to methods and compositions utilizing conserved genetic sequences of known hepatitis B viral (HBV) strains and known hepatitis C viral (HCV) strains to modulate the expression of HBV and/or HCV in mammalian cells, via double-stranded RNA-mediated gene silencing, including post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS).

BACKGROUND OF THE INVENTION

Human hepatitis C (HCV) is a major public health problem with an estimated 200 million persons worldwide infected. The number of new infections per year in the United States is estimated to be about 25,000 in 2001. This number has declined from an estimated 240,000 new cases per year in the 1980's due to blood donor screening. Nevertheless, an estimated 3.9 million (1.8%) Americans have been infected with HCV, of whom 2.7 million are chronically infected. Hepatitis C shows significant genetic variation in worldwide populations, evidence of its frequent rates of mutation and rapid evolution. There are six basic genotypes of HCV, with 15 recorded subtypes, which vary in prevalence across different regions of the world. Each of these major genotypes may differ significantly in their biological effects - in terms of replication, mutation rates, type and severity of liver damage, and detection and treatment options - however, these differences are not yet clearly understood.

There is currently no vaccine against HCV and available drug therapy, including ribavirin and interferon, is only partially effective. It is estimated that 75-85% of infected persons will develop a chronic infection, with 70% of chronically infected persons expected to develop chronic liver disease including hepatocellular carcinoma. Chronic HCV related liver disease is a leading indication for liver transplant.

Although a human hepatitis B vaccine has been available since 1982, it is estimated that 350 million persons worldwide are chronically infected with HBV. While the number of new infections per year in the United States has declined from an average of 260,000 in the 1980s to about 78,000 in 2001, there are an estimated 1.25 million hepatitis B carriers, defined as persons positive for hepatitis B surface antigen (HBsAg) for more than 6 months. Such carriers of HBV are at increased risk for developing cirrhosis, hepatic decompensation, and hepatocellular carcinoma. Although most carriers do not develop hepatic complications from chronic hepatitis B, 15% to 40% will develop serious sequelae during their lifetime, and death from chronic liver disease occurs in 15-25% of chronically infected persons.

There is a need for improved therapeutic agents effective in patients suffering from HBV and/or HCV infection, especially chronic infection, which together are estimated to account for 75% of all cases of liver disease around the world. There is also an extreme need for prophylactic methods and agents effective against HCV.

Nucleic acids (e.g., DNA, RNA, hybrid, heteroduplex, and modified nucleic acids) have come to be recognized as extremely valuable agents with significant and varied biological activities, including their use as therapeutic moieties in the prevention and/or treatment of disease states in man and animals. For example, oligonucleotides acting through antisense mechanisms are designed to hybridize to target mRNAs, thereby modulating the activity of the mRNA. Another approach to the utilization of nucleic acids as therapeutics is designed to take advantage of triplex or triple strand formation, in which a single-stranded oligomer (e.g., DNA or

RNA) is designed to bind to a double-stranded DNA target to produce a desired result, e.g., inhibition of transcription from the DNA target. Yet another approach to the utilization of nucleic acids as therapeutics is designed to take advantage of ribozymes, in which a structured RNA or a modified oligomer is designed to bind to an RNA or a double-stranded DNA target to produce a desired result, e.g., targeted cleavage of RNA or the DNA target and thus inhibiting its expression. Nucleic acids may also be used as immunizing agents, e.g., by introducing DNA molecules into the tissues or cells of an organism that express proteins capable of eliciting an immune response. Nucleic acids may also be engineered to encode an RNA with antisense, ribozyme, or triplex activities, or to produce RNA that is translated to produce protein(s) that have biological function.

More recently, the phenomenon of RNAi or double-stranded RNA (dsRNA)-mediated gene silencing has been recognized, whereby dsRNA complementary to a region of a target gene in a cell or organism inhibits expression of the target gene (see, e.g., WO 99/32619, published 1 July 1999, Fire et al.; and U.S. 6,506,559: "Genetic Inhibition by Double-Stranded RNA;" WO 00/63364: "Methods and Compositions for Inhibiting the Function of Polynucleotide Sequences," Pachuk and Satishchandran; and U.S.S.N. 60/419,532, filed October 18, 2002). dsRNA-mediated gene silencing, utilizing compositions providing an at least partially double-stranded dsRNA, is expected to provide extremely valuable therapeutic and/or prophylactic agents against viral infection, including HBV and/or HCV, including in the extremely difficult problem of chronic HBV and/or HCV infection.

SUMMARY OF THE INVENTION

Applicants' invention provides a method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide

sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. In a preferred embodiment of the
5 method, effector sequences from more than one SEQ ID sequence may be administered to the same cell, and/or more than one effector sequence from within the same SEQ ID sequence may be administered to the same cell.

Applicants' method further provides a method for inhibiting
10 expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering to said cell a double-stranded RNA effector molecule comprising comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is
15 substituted for T. In a preferred embodiment of this aspect of the method, effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 may be administered to the same cell; and/or more than one effector molecule from within the same SEQ ID NO may be administered to the same cell.

Applicants' method further provides A method for inhibiting
20 expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in the same *in vivo* mammalian cell, comprising administering to said cell a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group
25 consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group
30 consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T. In preferred embodiments of this aspect of the invention, effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10

may be administered to the same cell; and/or effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 may be administered to the same cell; and/or more than one effector molecules from within the same SEQ ID NO may be administered to the same cell.

- 5 Applicants' invention further provides a composition for inhibiting the expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO is present in the composition.
- 10 SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO is present in the composition.
- 15 Applicants' invention further provides a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO may be present in the composition.

- 20 Applicants' invention further provides a composition for inhibiting the expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in a single *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO may be present in the composition.
- 25 Applicants' invention further provides a composition for inhibiting the expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in a single *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO may be present in the composition.

- 30 Applicants' invention further provides a composition for inhibiting the expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in a single *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO may be present in the composition.

- NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence
- 5 from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein effector molecules from both SEQ ID NO:11
- 10 and SEQ ID NO:12 are present in the composition; and/or wherein more than one effector sequence from within the same SEQ ID NO may be present in the composition.

In particularly preferred embodiments of the above methods and compositions of the invention, the polynucleotide sequence is RNA, and

15 the mammalian cell is a human cell.

Further provided are compositions for inhibiting the expression of a polynucleotide sequence of hepatitis B virus and/or a polynucleotide sequence of hepatitis C virus in mammalian cells, wherein said compositions comprise an at least 19 contiguous nucleotide sequence

20 selected from within SEQ ID NO:1 through SEQ ID NO:12, the complement sequences of SEQ ID NO:1 through SEQ ID NO:12, and mixtures of these sequences. In this embodiment of the invention, the "an at least 19 contiguous nucleotide sequence" is preferably DNA, and the mammalian cell is preferably human. Also provided are expression

25 constructs comprising any of the aforementioned compositions and a mammalian cell comprising said expression constructs.

Another aspect of the invention provides for a polynucleotide sequence comprising a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for

30 polynucleotide sequence comprising nucleotides 1-19, 1-20, 1-21, 2-20, 2-21, or 3-21 of a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for a polynucleotide

sequence comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from SEQ ID NO:27 through SEQ ID NO:44.

Another aspect of the invention provides a composition for inhibiting
5 the expression of a polynucleotide sequence of hepatitis C virus in a mammalian cell, comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:27; wherein U is substituted for T.

10

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 through SEQ ID NO:10 represent conserved regions of the hepatitis B genome.

SEQ ID NO:11 and SEQ ID NO:12 represent conserved regions of the hepatitis C genome.

15 SEQ ID NO:13 represents the nucleotide sequence of human U6 promoter.

SEQ ID NO:14 and SEQ ID NO:15 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:5.

20 SEQ ID NO:16 and SEQ ID NO:17 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:4.

SEQ ID NO:18 represents eiRNA that has an HBV sequence mapping within SEQ ID NO:10.

SEQ ID NO:19 through SEQ ID NO:22 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:3.

25 SEQ ID NO:23 and SEQ ID NO:24 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:2.

SEQ ID NO:25 and SEQ ID NO:26 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:1.

SEQ ID NO:27 represents the "X" region of the HCV 3'UTR.

30 SEQ ID NO:28 through SEQ ID NO:36 represent siRNAs mapping to the the HCV 3'UTR.

SEQ ID NO:37 through SEQ ID NO:44 represent siRNAs mapping to the "X" region of the HCV 3'UTR.

SEQ ID NO:45 represents an siRNA mapping to the HCV core.

SEQ ID NO:46 represents an siRNA mapping to lamin.

5 SEQ ID NO:47 represents the T7 RNA polymerase gene.

SEQ ID NO:48 represents a T7-based eiRNA vector encoding a hairpin RNA.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Figure 1 depicts a vector illustrating placement of the T7 RNA polymerase promoter and T7 RNA polymerase, showing inclusion of hairpin eiRNA sequences.

15 Figure 2 is a graph showing HBsAg inhibition corresponding to data found in Table 2.

Figure 3 is a graph showing HBsAg inhibition corresponding to data found in Table 3.

20 Figure 4 is a graph showing HBsAg inhibition corresponding to data found in Table 4.

Figure 5 is a graph showing HBsAg inhibition corresponding to data found in Table 5.

25

Figure 6 is a graph showing HBsAg inhibition corresponding to data found in Table 6.

30 Figure 7 is a graph showing HBsAg inhibition corresponding to data found in Table 7.

Figure 8 is a graph showing HBsAg inhibition corresponding to data found in Table 8.

Figure 9 is a drawing depicting effective HBV-AYW shRNA inserts.
5

Figure 10 is a graph showing HBsAg inhibition corresponding to data found in Table 9.

Figure 11 is a bar graph showing downregulation of HBV RNA by
10 Northern Blot analysis.

Figure 12 is a graph showing HBsAg inhibition corresponding to data found in Table 12.

15 Figure 13 is a Western Blot showing levels of HCV NS5A protein at (I to r) 0, 9, and 20 pmole of the identified siRNAs, as described in more detail in Experiment 1 of Example 2.

20 Figure 14 is a Western Blot showing levels of HCV NS5A protein at (I to r) 0, 9, and 20 pmole of the identified siRNA, and 0, 3, and 9 pmole of the "core" positive control siRNA, as described in more detail in Experiment 2 of Example 2.

DETAILED DESCRIPTION OF THE INVENTION

25 RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing or transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Since RNA interference acts in a sequence specific manner, the RNAi molecule used as a drug must be specific to its target. Viral genomes are variable to accommodate resistance to changes in the environment. While HBV and HCV are very desirable viral targets for RNAi, the variability and mutability of the viruses
30

and the high rates of transcription of the viruses make HBV and HCV very challenging targets for any therapeutic and/or prophylactic approach. In order to knock down viral genome replication using RNAi there is a need to identify conserved and unique regions in the viral genome. At that 5 same time, it is very important in order to avoid toxicity that any sequences selected for gene silencing be absent from the human genome.

Human Hepatitis B Virus (HBV) Hepatitis B virus belongs to the family of hepadnaviruses. The HBV genome is a relaxed circular, partially double stranded DNA of approximately 3,200 base pairs. There are 4 10 partially overlapping open reading frames encoding the envelope (pre-S/S), core (precore/core), polymerase, and X proteins. The pre-S/S open reading frames encode the large (L), middle (M), and small (S) surface glycoproteins. The precore/core open reading frame is translated into a precore polypeptide, which is modified into a soluble protein, the hepatitis 15 B e antigen (HBeAg) and the nucleocapsid protein, hepatitis B core antigen. Mutations in the core promoter and precore region have been shown to decrease or abolish HBeAg production. The polymerase protein functions as a reverse transcriptase as well as a DNA polymerase. The X protein is a potent transactivator and may play a role in 20 hepatocarcinogenesis.

The replication cycle of HBV begins with the attachment of the virion to the hepatocyte. Inside the hepatocyte nucleus, synthesis of the plus strand HBV DNA is completed and the viral genome is converted into a covalently closed circular DNA (cccDNA). Most antiviral agents that have 25 been examined so far have little or no effect on cccDNA, which accounts for the rapid reappearance of serum HBV DNA after cessation of antiviral therapy. The aims of treatment of chronic hepatitis B are to achieve sustained suppression of HBV replication and/or expression of HBV antigens and remission of liver disease.

30 In GenBank version 132.0 there are more than 4500 HBV sequences and 340 HBV complete genome sequences (317 Human isolates, 22 isolates from other primates and one woodchuck HBV isolate).

This variability constitutes a serious challenge for sequence-specific pharmaceutical approaches such as RNAi. In order to identify conserved sequences suitable for RNAi applications, a comparison between all the complete genomes was carried out using a modified version of ClustalW.

- 5 Two multiple alignment schemes were generated: the first included all 339 HBV complete genome sequences and the second was limited to all Human HBV isolates. The multiple alignment results were parsed and a table that included scores for sequence conservation at each position in the HBV genome was generated. A sliding window search to identify the
10 longest region of sequence conservation larger than 19 nt in length was created. Three major conserved regions were identified and mapped to GenBank accession no.: AF090840, a Human HBV isolate. The conserved HBV sequences were screened against Genbank sequences of both human genomic and cDNA libraries (Human chromosomes database). It
15 was found that the identified conserved viral sequences are unique from 21 nt up. For human therapeutic purposes, assuring that homologous human sequences are not inadvertently silenced is as important as selecting conserved viral sequences for RNAi.

- Human Hepatitis C Virus HCV is a small (40 to 60 nanometers in diameter), enveloped, single-stranded RNA virus of the family Flaviviridae and genus hepacivirus. The genome is approximately 10,000 nucleotides and encodes a single polyprotein of about 3,000 amino acids, which is post-transcriptionally cleaved into 10 polypeptides, including 3 major structural (C, E1, and E2) and multiple non-structural proteins ([NS] NS2 to
25 NS5). The NS proteins include enzymes necessary for protein processing (proteases) and viral replication (RNA polymerase). Because the virus mutates rapidly, changes in the envelope proteins may help it evade the immune system. There are at least 6 major genotypes and more than 90 subtypes of HCV. The different genotypes have different geographic
30 distributions. Genotypes 1a and 1b are the most common in the United States (about 75 % of cases). Genotypes 2a and 2b (approximately 15%) and 3 (approximately 7%) are less common.

- There is little difference in the severity of disease or outcome of patients infected with different genotypes. However, patients with genotypes 2 and 3 are more likely to respond to interferon treatment. The virus replicates at a high rate in the liver and has marked sequence
- 5 heterogeneity. The main goal of treatment of chronic hepatitis C is to eliminate detectable viral RNA from the blood. Lack of detectable hepatitis C virus RNA from blood six months after completing therapy is known as a sustained response. Studies suggest that a sustained response is equated with a very favorable prognosis and that it may be equivalent to a cure.
- 10 There may be other more subtle benefits of treatment, such as slowing the progression of liver scarring (fibrosis) in patients who do not achieve a sustained response.

In GenBank version 134.0 there are more than 20,000 HCV sequences and 93 HCV complete genome sequences. A comparison

15 between all the complete genomes was carried out using a modified version of ClustalW. The multiple alignment result was parsed and a table that included scores for sequence conservation at each position in the HCV genome was generated. A sliding window search to identify the longest region of sequence conservation larger than 19 nt in length was

20 created. Three major conserved regions were identified and mapped to GenBank RefSeq (reference sequence) accession no.: NC_004102 this is GenBank annotated HCV complete genome. The conserved sequences were screened against Genbank sequences of both human genomic and cDNA libraries (human chromosomes database), and those sequences

25 unique from 21 nt up were identified.

Non-Homology with Human Sequences

It is equally important to ensure that conserved viral sequences targeted for silencing according to the invention be substantially non-

30 homologous to any naturally occurring, normally functioning, and essential human polynucleotide sequence, so that the dsRNA molecule does not adversely affect the function of any essential naturally occurring

mammalian polynucleotide sequence, when used in the methods of this invention. Such naturally occurring functional mammalian polynucleotide sequences include mammalian sequences that encode desired proteins, as well as mammalian sequences that are non-coding, but that provide for 5 essential regulatory sequences in a healthy mammal. Essentially, the RNA molecule useful in this invention must be sufficiently distinct in sequence from any mammalian polynucleotide sequence for which the function is intended to be undisturbed after any of the methods of this invention are performed. Computer algorithms may be used to define the essential lack 10 of homology between the RNA molecule polynucleotide sequence and the normal mammalian sequences.

Since the length of a contiguous dsRNA sequence capable of association with and activation of RISC (RNA-induced silencing complex), is generally considered to be 19-27 base pairs, the identified conserved 15 HBV and HCV sequences were compared with both human genomic libraries and, perhaps even more importantly, with human cDNA libraries. Since human cDNA libraries represent expressed sequences that appear in mRNAs, such mRNA sequences would be especially vulnerable to silencing by homologous dsRNA sequences provided to a cell.

20 Accordingly, the conserved HBV and HCV sequences were compared with human genomic and cDNA sequences. No human cDNA library matches to the HBV or HCV conserved sequences were identified. (Although there were some matches that were ultimately identified as HBV contamination in the cDNA library.) A comparison with human genomic 25 library sequences revealed no match of any sequence of 21 nts or more, one match of 20 nucleotides, and one match of 19 nucleotides. These matches were in non-coding regions, and likely do not appear in mRNA since cognates were not turned up in the cDNA library. Therefore, they are considered unlikely to be a safety risk, but could be excluded if desired.

Conserved sequences from HBV and HCVHBV Conserved Region 1

GAACATGGAGA[A(89%)/G(11%)]CA[T(76%)/C(24%)][C(78%)
5 %)/A(20%)/T(2%)][A(78%)/G(21%)/T(1%)]CATCAGGA[T(65%)/c(35%)]TC
CTAGGACCCCTGCTCGTGTACAGGC GG[G(88%)/t(12%)]GT[T(89%)/G
(11%)]TTTCT[T(94%)/C(6%)]GTTGACAA[G(64%)/A(36%)]AATCCTCACA
ATACC[A(56%)/G(43%)/T(1%)]CAGAGTCTAGACTCGTGGTGGACTTCT
CTCAATTCTAGGGGG[G(92%)/A(5%)/T(3%)]A[A(41%)/G(30%)/T(18%)/
C(11%)][C(90%)/T(10%)]

10

HBV Conserved Region 2

TGGATGTGTCT[G(99%)/A(1%)]CGGCGTTTATCAT

HBV Conserved Region 3

15 AAGGCCTTCT[A(43%)/G(43%)/C(14%)][T(56%)/A(37%)/C
(7%)]GT[A(87%)/C(13%)]AACAA[A(57%)/G(43%)]TA[T(59%)/C(41%)][C(59%)/A(41%)]TG[A(92%)/C(8%)][A(93%)/C(7%)]CCTTACCCCGTTGC[T(54%)/C(46%)][C(92%)/A(8%)]GGCAACGG[C(74%)/T(24%)]C[A(50%)/T(43%)/c(7%)]GG[T(87%)/C(13%)]CT[G(70%)/C(19%)/T(7%)/A(4%)]TGCCAA
20 GTGTTGCTGACGCAACCCCCACTGG[C(48%)/T(38%)/A(14%)]TGGGG
CTTGG[C(84%)/T(16%)][C(84%)/T(12%)/G(4%)]AT[A(47%)/T(23%)/G(17%)/C(13%)]GGCCATC[A(83%)/G(17%)][G(92%)/C(8%)]CGCATGCGTGG
AACCTTT[G(84%)/C(13%)/T(3%)][T(92%)/A(4%)/C(3%)/G(1%)]G[G(78%)/T(22%)]CTCCTCTGCCGATCCATACTGCGGAACCTCCT[A(88%)/T(9%)/G
25 (1%)/C(1%)]GC[C(57%)/A(35%)/T(6%)/G(2%)]GC[T(92%)/C(7%)/G(1%)]T
GT TT[T(88%)/C(12%)]GCTCGCAGC[C(64%)/A(36%)]GGTCTGG[A(87%)/
G(13%)]GC

HBV Conserved Region 4

30 [C(62%)/T(38%)]ACTGTTCAAGCCTCAAGCTGTGCCTTGG
GTGGCTTT[G(88%)/A(12%)]GG[G(92%)/A(8%)]CATGGACATTGAC[C(92%)/A(8%)]C[T(65%)/G(35%)]TATAAAGAATTGGAGCT[A(65%)/T(35%)]

CTGTGGAGTTACTCTC[G(62%)/T(35%)/A(3%)]TTTTTGCCTTC[T(92%)/C(8%)]GACTT[C(92%)/T(8%)]TTTCCTTC

HBV Conserved Region 5

5 [C(69%)/del(31%)][G(69%)/del(31%)]A[G(85%)/T(11%)/C(4%)][GCAGGTCCCCTAGAAGAAGAACTCCCTGCCTCGCAGACG[C(61%)/A(39%)]G[A(62%)/G(38%)]TCTCAATCG[C(88%)/A(12%)]CGCGTCGCAGAAGATCTCAAT[C(92%)/T(8%)]TCGGGAATCT[C(88%)/T(12%)]AATGTTAGTAT

10

HBV Conserved Region 6

TTGG[C(84%)/t(16%)][C(84%)/t(12%)/g(4%)]AT[A(47%)/t(23%)/g(17%)/c(13%)]GGCCATC[A(83%)/g(17%)][G(92%)/c(8%)]CGCATGC
15 GTGGAACCTTT[G(84%)/c(13%)/t(3%)][T(92%)/a(4%)/c(3%)/g(1%)]G[G(78%)/t(22%)]CTCCTCTGCCGATCCATACTGCGGAACTCCT[A(88%)/t(9%)/g(1%)/c(1%)]GC[C(57%)/a(35%)/t(6%)/g(2%)]GC[T(92%)/c(7%)/g(1%)]T
GTTT[T(88%)/c(12%)]GCTCGCAGC[C(64%)/a(36%)]GGTCTGG[A(87%)/g(13%)]GC

20

HBV Conserved Region 7

CTGCCAACTGGAT[C(86%)/T(10%)/A(4%)]CT[C(69%)/T(25%)/A(6%)]CGCGGGACGTCTTGT[T(75%)/C(25%)]TACGTCCCGTC[G(93%)/A(7%)]GCGCTGAATCC[C(86%)/T(7%)/A(7%)]GC GGACGACCC[C(52%)/G(25%)/T(19%)/A(4%)]

25

HCV Conserved Region 1

[A(74%)/G(19%)/T(7%)][G(82%)/A(15%)/T(3%)]ATCACTCC
CCTGTGAGGAACACTGTCTTCACGCAGAAAGCGTCTAGCCATGGCG
TTAGTATGAGTGT[C(92%)/T(7%)]GTGCAGC[C(89%)/T(10%)]TCCAGG[
30 A(76%)/T(14%)/C(8%)/G(1%)]CCCCCCCCTCCCGGGAGAGCCATAGTGG
TCTGCGGAACCGGTGAGTACACCGGAATTGCC[A(90%)/G(9%)]GGA[C
(78%)/T(16%)/A(5%)]GACC GGTCCTTCTTGAT[G(78%)/T(11%)/A(10

%)]AACCCGCTC[A(94%)/T(5%)]ATGCC[T(90%)/C(9%)]GGA[G(91%)/C(4%)
%/A(4%)]ATTGGCGTGCCTCGC[G(85%)/A(14%)]AGAC[T(94%)/C(5%)]GCTAGCCGAGTAG[T(92%)/C(7%)]GTTGGGT[C(94%)/T(5%)]GCGA
AAGGCCTTGTGGTACTGCCTGATAGGGTGCTGCGAGTGCCCCGGG
5 AGGTCTCGTAGACCGTGCA[C(62%)/T(30%)/A(8%)]CATGAGCAC[A(50%)/G(50%)][A(92%)/C(8%)][A(89%)/T(11%)]TCC[T(92%)/A(5%)/C(3%)]AA
ACC[T(84%)/C(14%)/A(2%)]CAAAGAAAAACCAAA[C(84%)/A(16%)]G[T(84%)/A(16%)]AACACCAACCG[C(77%)/T(23%)]CGCCCACAGGACGT[C(81%)/T(18%)/A(1%)]AAGTTCCCAGG[C(89%)/T(11%)]GG[T(80%)/C(20%)]
10 GG[T(80%)/C(17%)/A(3%)]CAGATCGTTGG[T(91%)/C(8%)/G(1%)]GGAG
T[T(87%)/A(11%)/C(2%)]TAC[C(74%)/T(20%)/G(6%)]TGTGCCGCGCAG
GGGCC[C(87%)/T(8%)/A(4%)/G(1%)][A(92%)/C(8%)][G(92%)/A(5%)/C(2%)][G(87%)/A(12%)/T(1%)]TTGGGTGTGCGCGAC[T(78%)/G(13%)/A
(7%)/C(2%)]AGGAAGACTTC[C(90%)/G(5%)/T(5%)]GA[G(90%)/A(10%)]C
15 GGTC[G(79%)/C(12%)/A(8%)/T(1%)]CA[A(86%)/G(14%)]CC[T(88%)/A(6%)]C(6%)]CG[T(82%)/C(9%)]GG[A(87%)/T(8%)/G(3%)/C(2%)]AG

HCV Conserved Region 2

ATGGC[T(76%)/A(12%)/C(10%)/G(2%)]TGGGATATGATGA
20 TGAACTGG[T(81%)/C(19%)]C

Conserved Consensus Sequences presented in SEQ ID format

The following sequences are presented in the format required per
the WIPO Standard ST.25 (1998), using the codes provided under 37 CFR
25 1.821. SEQ ID NO:1 through SEQ ID NO:10 are derived from the HBV
genome SEQ ID NO:11 and SEQ ID NO:12 are derived from the HCV
genome.

SEQ ID NO:1 HBV

30 GAACATGGAGArCAyhdCATCAGGAYTCCTAGGACCCCT
GCTCGTGTACAGGCGGkGTkTTCTyGTTGACAARAAATCCTCACAAATA

CCdCAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTCTAGGGGdA
ny

SEQ ID NO:2 HBV
5 TGGATGTGTCTrCGGCCTTTATCAT

SEQ ID NO:3 HBV
AAGGCCTTCTvhGTmAACArTAymTGmmCCTTACCCC
GTTGCymGGCAACGGyChGGyCTnGCCAAGTGTGCTGACGCAAC
10 CCCCCACTGGhTGGGGCTTGGybATnGCCATCrscGCATGCGTGGAA
CCTTTbnGkCTCCTCTGCCGATCCATACTGCGGAACTCCTnGCnGCbT
GTTTyGCTCGCAGCmGGTCTGGGrGC

SEQ ID NO:4 HBV
15 yACTGTTCAAGCCTCAAGCTGTGCCTGGGTGGCTTTrG
GrCATGGACATTGACmCkTATAAAGAATTGGAGCTwCTGTGGAGTTA
CTCTCdTTTTGCCTCyGACTTyTTTCCTTC

SEQ ID NO:5 HBV
20 CGAbGCAGGTCCCTAGAAGAAGAACTCCCTGCCCTCG
CAGACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGA
ATCTyAATGTTAGTAT

SEQ ID NO:6 HBV
25 AbGCAGGTCCCTAGAAGAAGAACTCCCTGCCCTCGCA
GACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAAT
CTyAATGTTAGTAT /

SEQ ID NO:7 HBV
30 CAAbGCAGGTCCCTAGAAGAAGAACTCCCTGCCCTCGC
AGACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGA
TCTyAATGTTAGTAT

SEQ ID NO:8 HBV
GAbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCCTCGC
AGACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAA
5 TCTyAATGTTAGTAT

SEQ ID NO:9 HBV
TTGGybATnGCCATCrsgcATGCGTGGAACCTTTbnGk
CTCCTCTGCCGATCCATACTGCGGAACCTCCTnGCnGCbTGTTTyGCTC
10 GCAGCmGGTCTGGrGC

SEQ ID NO:10 HBV
CTGCCAACTGGATHCThCGCGGACGTCCCTTGTyTACG
TCCCGTCrGCGCTGAATCChGCGGACGACCCn
15

SEQ ID NO:11 HCV
DdATCACTCCCCGTGAGGAACACTACTGTCTTCACGCAGA
AAGCGTCTAGCCATGGCGTTAGTATGAGTGTyGTGCAGCyTCCAGGn
CCCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTA
20 CACCGGAATTGCCrGGAhGACCGGGTCCTTCTTGGATdAACCGCT
CwATGCCyGGAvATTGGGCGTGCCCCCGCrAGACyGCTAGCCGAGT
AGyGTTGGGTyGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTT
GCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCAhCATGAGCACrmw
TCChAAACChCAAAGAAAAACCAAmGwAACACCAACCGyCGCCCAC
25 AGGACGThAAGTTCCCGGGyGGyGGhCAGATCGTTGGbGGAGThTAC
bTGTGCGCGCAGGGGCCnmvdTTGGGTGTGCGCGACnAGGA
AGACTTCbGArCGGTCnCArCChCGhGGnAG

SEQ ID NO:12 HCV
30 ATGGCnTGGGATATGATGATGAACCTGGyC

- *Double Stranded RNA Gene Silencing/RNAi
- By "nucleic acid composition" or "nucleotide" composition is meant any one or more compounds in which one or more molecules of phosphoric acid are combined with a carbohydrate (e.g., pentose or hexose) which are in turn combined with bases derived from purine (e.g., adenine) and from pyrimidine (e.g., thymine). Particular naturally occurring nucleic acid molecules include genomic deoxyribonucleic acid (DNA) and host ribonucleic acid (RNA), as well as the several different forms of the latter, e.g., messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Also included are different DNA molecules which are complementary (cDNA) to the different RNA molecules. Synthesized DNA or a hybrid thereof with naturally occurring DNA, as well as DNA/RNA hybrids, and peptide nucleic acid (PNA) molecules (Gambari, Curr Pharm Des 2001 Nov;7(17):1839-62) can also be used.
- It is contemplated that where the desired nucleic acid molecule is RNA, the T (thymine) in the sequences provided herein is substituted with U (uracil). For example, SEQ ID NO:1 through SEQ ID NO:44 are disclosed herein as DNA sequences. It will be obvious to one of ordinary skill in the art that an RNA effector molecule comprising sequences from any of the aforementioned SEQ ID NOs will have T substituted with U.
- Nucleic acids typically have a sequence of two or more covalently bonded naturally-occurring or modified deoxyribonucleotides or ribonucleotides. Modified nucleic acids include, e.g., peptide nucleic acids and nucleotides with unnatural bases.
- By "dsRNA" is meant a nucleic acid containing a region of two or more nucleotides that are in a double stranded conformation. It is envisioned that the conserved viral sequences of the invention may be utilized in any of the many compositions known in the art or subsequently developed which act through a dsRNA-mediated gene silencing or RNAi mechanism. In various embodiments, the dsRNA consists entirely of ribonucleotides or consists of a mixture of ribonucleotides and deoxynucleotides, such as the RNA/DNA hybrids disclosed, for example,

by WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. The dsRNA may be a single molecule with a region of self-complementarity such that nucleotides in one segment of the molecule base pair with nucleotides in another segment of the molecule. In various 5 embodiments, a dsRNA that consists of a single molecule consists entirely of ribonucleotides or includes a region of ribonucleotides that is complementary to a region of deoxyribonucleotides. Alternatively, the dsRNA may include two different strands that have a region of complementarity to each other. In various embodiments, both strands 10 consist entirely of ribonucleotides, one strand consists entirely of ribonucleotides and one strand consists entirely of deoxyribonucleotides, or one or both strands contain a mixture of ribonucleotides and deoxyribonucleotides. Desirably, the regions of complementarity are at least 70, 80, 90, 95, 98, or 100% complementary. Desirably, the region of 15 the dsRNA that is present in a double stranded conformation includes at least 19, 20, 21, 22, 23, 24, 30, 50, 75, 100, 200, 500, 1000, 2000 or 5000 nucleotides or includes all of the nucleotides in a cDNA being represented in the dsRNA. In some embodiments, the dsRNA does not contain any single stranded regions, such as single stranded ends, or the dsRNA is a 20 hairpin. In other embodiments, the dsRNA has one or more single stranded regions or overhangs. Desirable RNA/DNA hybrids include a DNA strand or region that is an antisense strand or region (e.g., has at least 70, 80, 90, 95, 98, or 100% complementarity to a target nucleic acid) and an RNA strand or region that is a sense strand or region (e.g., has at 25 least 70, 80, 90, 95, 98, or 100% identity to a target nucleic acid). In various embodiments, the RNA/DNA hybrid is made *in vitro* using enzymatic or chemical synthetic methods such as those described herein or those described in WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. In other embodiments, a DNA strand 30 synthesized *in vitro* is complexed with an RNA strand made *in vivo* or *in vitro* before, after, or concurrent with the transformation of the DNA strand into the cell. In yet other embodiments, the dsRNA is a single circular

nucleic acid containing a sense and an antisense region, or the dsRNA includes a circular nucleic acid and either a second circular nucleic acid or a linear nucleic acid (see, for example, WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999.) Exemplary circular nucleic acids include lariat structures in which the free 5' phosphoryl group of a nucleotide becomes linked to the 2' hydroxyl group of another nucleotide in a loop back fashion.

In other embodiments, the dsRNA includes one or more modified nucleotides in which the 2' position in the sugar contains a halogen (such as fluorine group) or contains an alkoxy group (such as a methoxy group) which increases the half-life of the dsRNA *in vitro* or *in vivo* compared to the corresponding dsRNA in which the corresponding 2' position contains a hydrogen or an hydroxyl group. In yet other embodiments, the dsRNA includes one or more linkages between adjacent nucleotides other than a naturally-occurring phosphodiester linkage. Examples of such linkages include phosphoramido, phosphorothioate, and phosphorodithioate linkages. The dsRNAs may also be chemically modified nucleic acid molecules as taught in U.S. Patent No. 6,673,661. In other embodiments, the dsRNA contains one or two capped strands, as disclosed, for example, by WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. In other embodiments, the dsRNA contains coding sequence or non-coding sequence, for example, a regulatory sequence (e.g., a transcription factor binding site, a promoter, or a 5' or 3' untranslated region (UTR) of an mRNA). Additionally, the dsRNA can be any of the at least partially dsRNA molecules disclosed in WO 00/63364, filed April 19, 2000 (see, for example, pages 8-22), as well as any of the dsRNA molecules described in US Provisional Application 60/399,998 filed July 31, 2002, and PCT/US2003/024028, filed 31-Jul-2003; and US Provisional Application 60/419,532 filed October 18, 2002, and PCT/US2003/033466, filed 20-Oct-2003, the teaching of which is hereby incorporated by reference. Any of the dsRNAs may be expressed *in vitro* or *in vivo* using the methods described herein or standard methods, such as those

described in WO 00/63364, filed April 19, 2000 (see, for example, pages 16-22).

dsRNA "Hairpin" Constructs: Constructs encoding a unimolecular hairpin dsRNA are more desirable for some applications than constructs 5 encoding duplex dsRNA (i.e., dsRNA composed of one RNA molecule with a sense region and a separate RNA molecule with an antisense region) because the single-stranded RNA with inverted repeat sequences more efficiently forms a dsRNA hairpin structure. This greater efficiency is due in part to the occurrence of transcriptional interference arising in 10 vectors containing converging promoters that generate duplex dsRNA. Transcriptional interference results in the incomplete synthesis of each RNA strand thereby reducing the number of complete sense and antisense strands that can base-pair with each other and form duplexes. Transcriptional interference can be overcome, if desired, through the use 15 of (i) a two vector system in which one vector encodes the sense RNA and the second vector encodes the antisense RNA, (ii) a bicistronic vector in which the individual strands are encoded by the same plasmid but through the use of separate cistrons, or (iii) a single promoter vector that encodes a hairpin dsRNA, i.e., an RNA in which the sense and antisense 20 sequences are encoded within the same RNA molecule. Hairpin-expressing vectors have some advantages relative to the duplex vectors. For example, in vectors that encode a duplex RNA, the RNA strands need to find and base-pair with their complementary counterparts soon after 25 transcription. If this hybridization does not happen, the individual RNA strands diffuse away from the transcription template and the local concentration of sense strands with respect to antisense strands is decreased. This effect is greater for RNA that is transcribed intracellularly compared to RNA transcribed *in vitro* due to the lower levels of template per cell. Moreover, RNA folds by nearest neighbor rules, resulting in RNA 30 molecules that are folded co-transcriptionally (i.e., folded as they are transcribed). Some percentage of completed RNA transcripts is therefore unavailable for base-pairing with a complementary second RNA because

- of intra-molecular base-pairing in these molecules. The percentage of such unavailable molecules increases with time following their transcription. These molecules may never form a duplex because they are already in a stably folded structure. In a hairpin RNA, an RNA sequence is
- 5 always in close physical proximity to its complementary RNA. Since RNA structure is not static, as the RNA transiently unfolds, its complementary sequence is immediately available and can participate in base-pairing because it is so close. Once formed, the hairpin structure is predicted to be more stable than the original non-hairpin structure. Especially desirable
- 10 are, e.g., "forced" hairpin constructs, partial hairpins capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002; and PCT/US2003/024028, "Double Stranded RNA Structures and Constructs and Methods for Generating and Using the Same," filed 31-Jul-2003; as well as the
- 15 "udderly" structured hairpins, hairpins with mismatched regions, and multiepitope constructs as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, "Double-Stranded RNA Structures and Constructs, and Methods for Generating and Using the Same," filed 20-Oct-2003.
- 20 By "short dsRNA" is meant a dsRNA that has about 50, 45, 40, 35, 30, 27, 25, 23, 21, 20 or 19 contiguous nucleotides in length that are in a double stranded conformation. Desirably, the short dsRNA is at least 19 basepairs in length. In desirable embodiments, the double stranded region is between 19 to 50, 19 to 40, 19 to 30, 19 to 25, 20 to 25, 21 to 23,
- 25 25 to 30, or 30 to 40 contiguous basepairs in length, inclusive. In some embodiments, the short dsRNA is between 30 to 50, 50 to 100, 100 to 200, 200 to 300, 400 to 500, 500 to 700, 700 to 1000, 1000 to 2000, or 2000 to 5000 nucleotides in length, inclusive and has a double stranded region that is between 38 and 60 contiguous basepairs in length, inclusive.
- 30 In one embodiment, the short dsRNA is completely double stranded. In some embodiments, the short dsRNA is between 11 and 30 nucleotides in length, and the entire dsRNA is double stranded. In other embodiments,

the short dsRNA has one or two single stranded regions. In particular embodiments, the short dsRNA binds PKR or another protein in a dsRNA-mediated stress response pathway. Desirably, the short dsRNA inhibits the dimerization and activation of PKR by at least 20, 40, 60, 80, 90, or 5 100%. In some desirable embodiments, the short dsRNA inhibits the binding of a long dsRNA to PKR or another component of a dsRNA-mediated stress response pathway by at least 20, 40, 60, 80, 90, or 100%. See also the teaching of USSN 10/425,006, filed 28-Apr-2003, "Methods of Silencing Genes Without Inducing Toxicity", Pachuk, as to utilization of 10 short dsRNAs in conjunction with other dsRNAs to avoid dsRNA-mediated toxicity.

By "at least 19 contiguous base pair nucleotide sequence" is meant that a nucleotide sequence can start at any nucleotide within one of the disclosed sequences, so long as the start site is capable of producing a 15 polynucleotide of at least 19 base pairs. For example, an at least 19 contiguous base pair nucleotide sequence can comprise nucleotide 1 through nucleotide 19, nucleotide 2 through nucleotide 20, nucleotide 3 through nucleotide 21, and so forth to produce a 19mer. Thus, a 20mer can comprise nucleotide 1 through nucleotide 20, nucleotide 2 through 20 nucleotide 21, nucleotide 3 through nucleotide 22, and so forth. Similar sequences above 20 contiguous nucleotides are envisioned.

By "expression vector" is meant any double stranded DNA or double stranded RNA designed to transcribe an RNA, e.g., a construct that contains at least one promoter operably linked to a downstream gene or 25 coding region of interest (e.g., a cDNA or genomic DNA fragment that encodes a protein, or any RNA of interest, optionally, e.g., operatively linked to sequence lying outside a coding region, an antisense RNA coding region, a dsRNA coding region, or RNA sequences lying outside a coding region). Transfection or transformation of the expression vector 30 into a recipient cell allows the cell to express RNA or protein encoded by the expression vector. An expression vector may be a genetically engineered plasmid, virus, or artificial chromosome derived from, for

example, a bacteriophage, adenovirus, retrovirus, poxvirus, or herpesvirus.

By an "expression construct" is meant any double-stranded DNA or double-stranded RNA designed to transcribe an RNA, e.g., a construct
5 that contains at least one promoter operably linked to a downstream gene or coding region of interest (e.g., a cDNA or genomic DNA fragment that encodes a protein, or any RNA of interest). Transfection or transformation of the expression construct into a recipient cell allows the cell to express RNA or protein encoded by the expression construct. An expression
10 construct may be a genetically engineered plasmid, virus, or artificial chromosome derived from, for example, a bacteriophage, adenovirus, retrovirus, poxvirus, or herpesvirus. An expression construct does not have to be replicable in a living cell, but may be made synthetically.

By "operably linked" is meant that a nucleic acid molecule and one
15 or more regulatory sequences (e.g., a promoter) are connected in such a way as to permit transcription of the mRNA or permit expression and/or secretion of the product (i.e., a polypeptide) of the nucleic acid molecule when the appropriate molecules are bound to the regulatory sequences.

By a "promoter" is meant a nucleic acid sequence sufficient to direct
20 transcription of a covalently linked nucleic acid molecule. Also included in this definition are those transcription control elements (e.g., enhancers) that are sufficient to render promoter-dependent gene expression controllable in a cell type-specific, tissue-specific, or temporal-specific manner, or that are inducible by external signals or agents; such elements,
25 which are well-known to skilled artisans, may be found in a 5' or 3' region of a gene or within an intron. Desirably a promoter is operably linked to a nucleic acid sequence, for example, a cDNA or a gene in such a way as to permit expression of the nucleic acid sequence.

The RNA molecule according to this invention may be delivered to
30 the mammalian or extracellular pathogen present in the mammalian cell in the composition as an RNA molecule or partially double stranded RNA sequence, or RNA/DNA hybrid, which was made *in vitro* by conventional

enzymatic synthetic methods using, for example, the bacteriophage T7, T3 or SP6 RNA polymerases according to the conventional methods described by such texts as the Promega Protocols and Applications Guide, (3rd ed. 1996), eds. Doyle, ISBN No. 1 57 Alternatively these molecules

5 may be made by chemical synthetic methods *in vitro* [see, e.g., Q. Xu et al., Nucleic Acids Res., 24(18):3643-4 (Sept. 1996); N. Naryshkin et al., Bioorg. Khim., 22(9):691-8 (Sept. 1996); J. A. Grasby et al., Nucleic Acids Res., 21(19):4444-50 (Sept 1993); C. Chaix et al., Nucleic Acids Res. 17:7381-93 (1989); S.H. Chou et al., Biochem., 28(6):2422-35 (Mar.

10 1989); O. Odal el al., Nucleic Acids Symp. Ser., 21:105-6 (1989); N.A. Naryshkin et al., Bioorg. Khim, 22(9):691-8 (Sept. 1996); S. Sun et al., RNA, 3(11):1352-1363 (Nov. 1997); X. Zhang et al., Nucleic Acids Res., 25(20):3980-3 (Oct. 1997); S. M. Grvaznov el al., Nucleic Acids Res., 2-6 15 (18):4160-7 (Sept. 1998); M. Kadokura et al., Nucleic Acids Symp. Ser., 37:77-8 (1997); A. Davison et al., Biomed. Pept. Proteins. Nucleic Acids, 2(I):1-6 (1996); and A. V. Mudrakovskaia et al., Bioorg. Khirn., 17(6):819-22 (Jun. 1991)].

Still alternatively, the RNA molecule of this invention can be made in a recombinant microorganism, e.g., bacteria and yeast or in a

20 recombinant host cell, e.g., mammalian cells, and isolated from the cultures thereof by conventional techniques. See, e.g., the techniques described in Sambrook et al, MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, which is exemplary of laboratory manuals that

25 detail these techniques, and the techniques described in US Patent Nos. 5,824,538; 5,877,159; and 5,643,771, incorporated herein by reference.

Such RNA molecules prepared or synthesized *in vitro* may be directly delivered to the mammalian cell or to the mammal as they are made *in vitro*. The references above provide one of skill in the art with the

30 techniques necessary to produce any of the following specific embodiments, given the teachings provided herein. Therefore, in one

embodiment, the "agent" of the composition is a duplex (i.e., it is made up of two strands), either complete or partially double stranded RNA.

In another embodiment, the agent is a single stranded RNA sense strand. In another embodiment, the agent of the composition is a single
5 stranded RNA anti-sense strand.

Preferably the single stranded RNA sense or anti-sense strand forms a hairpin at one or both termini. Desirably, the single stranded RNA sense or anti-sense strand forms a hairpin at some intermediate portion between the termini. Such a single stranded RNA sense or anti-sense
10 strand may also be designed to fold back upon itself to become partially double stranded *in vitro* or *in vivo*. Yet another embodiment of an extant RNA molecule as the effective agent used in the compositions is a single stranded RNA sequence comprising both a sense polynucleotide sequence and an antisense polynucleotide sequence, optionally separated
15 by a non-base paired polynucleotide sequence. Preferably, this single stranded RNA sequence has the ability to become double-stranded once it is in the cell, or *in vitro* during its synthesis.

Still another embodiment of this invention is an RNA/DNA hybrid as described above.

20 Still another embodiment of the synthetic RNA molecule is a circular RNA molecule that optionally forms a rod structure [see, e.g., K-S. Wang et al., Nature 323:508-514 (1986)] or is partially double-stranded, and can be prepared according to the techniques described in S. Wang et al., Nucleic Acids Res., 22(12):2326-33 (June 1994); Y. Matsumoto et al.,
25 Proc. Natl. Acad. Sci., USA, 87(19):7628-32 (Oct. 1990); E. Ford & M. Ares, Proc. Natl. Acad. Sci. USA 91(8):3117-21 (Apr. 1994); M. Tsagris et al., Nucleic Acids Res., 19 7):1605-12 (Apr. 1991); S. Braun et al., Nucleic Acids Res. 24(21):4152-7 (Nov. 1996); Z. Pasman et al., RNA, 2(6):603-10 (Jun. 1996); P. G. Zaphiropoulos, Proc. Natl. Acad. Sci., USA,
30 93(13):6536-41 (Jun. 1996); D. Beaudry et al., Nucleic Acids Res., 23(15):3064-6 (Aug. 1995), all incorporated herein by reference. Still

another agent is a double-stranded molecule comprised of RNA and DNA present on separate strands, or interspersed on the same strand.

- Alternatively, the RNA molecule may be formed *in vivo* and thus delivered by a “delivery agent” which generates such a partially double-stranded RNA molecule *in vivo* after delivery of the agent to the mammalian cell or to the mammal. Thus, the agent which forms the composition of this invention is, in one embodiment, a double stranded DNA molecule “encoding” one of the above-described RNA molecules. The DNA agent provides the nucleotide sequence which is transcribed 10 within the cell to become a double stranded RNA. In another embodiment, the DNA sequence provides a deoxyribonucleotide sequence which within the cell is transcribed into the above-described single stranded RNA sense or anti-sense strand, which optionally forms a hairpin at one or both termini or folds back upon itself to become partially double stranded. The DNA 15 molecule which is the delivery agent of the composition can provide a single stranded RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide sequence, optionally separated by a nonbase paired polynucleotide sequence, and wherein the single stranded RNA sequence has the ability to become double-stranded. 20 Alternatively, the DNA molecule which is the delivery agent provides for the transcription of the above-described circular RNA molecule that optionally forms a rod structure or partial double strand *in vivo*. The DNA molecule may also provide for the *in vivo* production of an RNA/DNA hybrid as described above, or a duplex containing one RNA strand and 25 one DNA strand. These various DNA molecules may be designed by resort to conventional techniques such as those described in Sambrook, cited above or in the Promega reference, cited above.

- A latter delivery agent of the present invention, which enables the formation in the mammalian cell of any of the above-described RNA 30 molecules, can be a DNA single stranded or double stranded plasmid or vector. Expression vectors designed to produce RNAs as described herein *in vitro* or *in vivo* may contain sequences under the control of any RNA

polymerase, including mitochondrial RNA polymerase, RNA pol I, RNA pol II, and RNA pol III, and viral polymerases, and bacteriophage polymerases such as T7 and Sp6. These vectors can be used to transcribe the desired RNA molecule in the cell according to this invention.

- 5 Vectors may be desirably designed to utilize an endogenous mitochondrial RNA polymerase (e.g., human mitochondrial RNA polymerase, in which case such vectors may utilize the corresponding human mitochondrial promoter). Mitochondrial polymerases may be used to generate capped (through expression of a capping enzyme) or uncapped messages *in vivo*.
- 10 RNA pol I, RNA pol II, and RNA pol III transcripts may also be generated *in vivo*. Such RNAs may be capped or not, and if desired, cytoplasmic capping may be accomplished by various means including use of a capping enzyme such as a vaccinia capping enzyme or an alphavirus capping enzyme. However, all pol II transcripts are capped. The DNA
- 15 vector is designed to contain one of the promoters or multiple promoters in combination (mitochondrial, RNA pol I, pol II, or pol III, or viral, bacterial or bacteriophage promoters along with the cognate polymerases). Preferably, where the promoter is RNA pol II, the sequence encoding the RNA molecule has an open reading frame greater than about 300 nts and must
- 20 follow the rules of design to prevent nonsense-mediated degradation in the nucleus. Such plasmids or vectors can include plasmid sequences from bacteria, viruses or phages.

- Such vectors include chromosomal, episomal and virus-derived vectors e.g., vectors derived from bacterial plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses, vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids and phagemids.
- 25

- Thus, one exemplary vector is a single or double-stranded phage vector. Another exemplary vector is a single or double-stranded RNA or
- 30 DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. The vectors, in the case of phage and viral

vectors may also be and preferably are introduced into cells as packaged or encapsidated virus by well known techniques for infection and transduction. Viral vectors may be replication competent or replication defective. In the latter case, viral propagation generally occurs only in 5 complementing host cells.

In another embodiment the delivery agent comprises more than a single DNA or RNA plasmid or vector. As one example, a first DNA plasmid can provide a single stranded RNA sense polynucleotide sequence as described above, and a second DNA plasmid can provide a 10 single stranded RNA anti-sense polynucleotide sequence as described above, wherein the sense and anti-sense RNA sequences have the ability to base-pair and become double-stranded. Such plasmid(s) can comprise other conventional plasmid sequences, e.g., bacterial sequences such as the well-known sequences used to construct plasmids and vectors for 15 recombinant expression of a protein. However, it is desirable that the sequences which enable protein expression, e.g., Kozak regions, etc., are not included in these plasmid structures.

The vectors designed to produce dsRNAs of the invention may desirably be designed to generate two or more, including a number of 20 different dsRNAs homologous and complementary to a target sequence. This approach is desirable in that a single vector may produce many, independently operative dsRNAs rather than a single dsRNA molecule from a single transcription unit and by producing a multiplicity of different dsRNAs, it is possible to self select for optimum effectiveness. Various 25 means may be employed to achieve this, including autocatalytic sequences as well as sequences for cleavage to create random and/or predetermined splice sites.

Other delivery agents for providing the information necessary for formation of the above-described desired RNA molecules in the 30 mammalian cell include live, attenuated or killed, inactivated recombinant bacteria which are designed to contain the sequences necessary for the required RNA molecules of this invention. Such recombinant bacterial

cells, fungal cells and the like can be prepared by using conventional techniques such as described in US Patent Nos. 5,824,538; 5,877,159; and 5,643,771, incorporated herein by reference. Microorganisms useful in preparing these delivery agents include those listed in the above cited 5 reference, including, without limitation, *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various species of *Pseudomonas*, *Streptomyces*, and *Staphylococcus*.

Still other delivery agents for providing the information necessary for formation of the desired, above-described RNA molecules in the 10 mammalian cell include live, attenuated or killed, inactivated viruses, and particularly recombinant viruses carrying the required RNA polynucleotide sequence discussed above. Such viruses may be designed similarly to recombinant viruses presently used to deliver genes to cells for gene therapy and the like, but preferably do not have the ability to express a 15 protein or functional fragment of a protein. Among useful viruses or viral sequences which may be manipulated to provide the required RNA molecule to the mammalian cell *in vivo* are, without limitation, alphavirus, adenovirus, adeno associated virus, baculoviruses, delta virus, pox viruses, hepatitis viruses, herpes viruses, papova viruses (such as SV40), 20 poliovirus, pseudorabies viruses, retroviruses, lentiviruses, vaccinia viruses, positive and negative stranded RNA viruses, viroids, and virusoids, or portions thereof. These various viral delivery agents may be designed by applying conventional techniques such as described in M. Di Nocla et al., *Cancer Gene Ther.*, 5(6):350-6 (1998), among others, with 25 the teachings of the present invention.

The term "*in vivo*" is intended to include any system wherein the cellular DNA or RNA replication machinery is intact, including tissue culture systems, and within single cell or multicellular living organisms.

By "multiple sequitope dsRNA" or "multisequitope dsRNA" is 30 meant an RNA molecule that has segments derived from multiple target nucleic acids or that has non-contiguous segments from the

- same target nucleic acid. For example, the multiple sequitope dsRNA may have segments derived from (i) sequences representing multiple genes of a single organism; (ii) sequences representing one or more genes from a variety of different organisms; and/or (iii)
- 5 sequences representing different regions of a particular gene (e.g., one or more sequences from a promoter and one or more sequences from an mRNA. Desirably, each segment has substantial sequence identity to the corresponding region of a target nucleic acid. In various desirable embodiments, a segment with substantial
- 10 sequence identity to the target nucleic acid is at least 19, 20, 21, 22, 23, 24, 30, 40, 50, 100, 200, 500, 750, or more basepairs in length. In desirable embodiments, the multiple epitope dsRNA inhibits the expression of at least 2, 4, 6, 8, 10, 15, 20, or more target genes by at least 20, 40, 60, 80, 90, 95, or 100%. In some embodiments, the
- 15 multiple epitope dsRNA has non-contiguous segments from the same target gene that may or may not be in the naturally occurring 5' to 3' order of the segments, and the dsRNA inhibits the expression of the nucleic acid by at least 50, 100, 200, 500, or 1000% more than a dsRNA with only one of the segments.
- 20 By "sequitope" is meant a contiguous sequence of double-stranded polyribonucleotides that can associate with and activate RISC (RNA-induced silencing complex), usually a contiguous sequence of between 19 and 27 basepairs, inclusive. Sequences comprising at least one sequitope from within one or more of the
- 25 conserved HBV and/or HCV nucleotide sequences identified here may be utilized for dsRNA mediated gene silencing as taught herein.
- 30 Multiple-epitope dsRNAs The advantages of a multiple-epitope or multisequitope double-stranded RNA approach as taught in USSN 60/419,532, filed 18-Oct-2002 and PCT/US2003/033466, filed 20-Oct-2003, are applicable to utilization of the conserved HBV and/or HCV

sequences of the invention. Because a singular species of dsRNA can simultaneously silence many target genes (e.g., genes from multiple pathogens, multiple genes or sequences from a single pathogen, or genes associated with multiple diseases), a multiple epitope dsRNA can be used

5 for many different indications in the same subject or used for a subset of indications in one subject and another subset of indications in another subject. For such applications, the ability to express long dsRNA molecules (e.g., dsRNA molecules with sequences from multiple genes) without invoking the dsRNA stress response is highly desirable. For

10 example, by using a series of sequences, each, e.g., as short as 19-21 nucleotides, desirably 100 to 600 nucleotides, or easily up to 1, 2, 3, 4, 5, or more kilobases such that the total length of such sequences is within the maximum capacity of the selected plasmid (e.g., 20 kilobases in length), a single such pharmaceutical composition can provide protection

15 against a large number of pathogens and/or toxins at a relatively low cost and low toxicity, e.g., HBV, HCV, HIV, etc.

The use of multiple epitopes derived from one or more genes from multiple strains and/or variants of a highly variable or rapidly mutating pathogen such as HBV and/or HCV can also be very advantageous. For

20 example, a singular dsRNA species that recognizes and targets multiple strains and/or variants of HBV and/or HCV can be used as a universal treatment or vaccine for the various strains/variants of HBV and/or HCV.

The ability to silence multiple genes of a particular pathogen such as HBV and/or HCV prevents the selection of, in this case, HBV and/or

25 HCV "escape mutants." In contrast, typical small molecule treatment or vaccine therapy that only targets one gene or protein results in the selection of pathogens that have sustained mutations in the target gene or protein and the pathogen thus becomes resistant to the therapy. By simultaneously targeting a number of genes or sequences of the pathogen

30 and or extensive regions of the pathogen using the multiple epitope approach of the present invention, the emergence of such "escape mutants" is effectively precluded.

- For example, it is considered particularly advantageous to include a mixture of sequences from both HCV SEQ ID NO:11 and SEQ ID NO:12, i.e., one or more sequences from HCV SEQ ID NO:11 together with one or more sequences from HCV SEQ ID NO:12, either in a single dsRNA
- 5 construct, an admixture of constructs, or through concomitant administration of such constructs to a patient, in order to decrease the ability of the virus to generate viable escape mutants. Similarly, it would be advantageous to provide a mixture of the conserved HBV sequences, in some cases in combination with one or more of the conserved HCV
- 10 sequences of the invention.
- Similarly, it may be desirable to use sequences from two or more of HBV SEQ ID NO:1, SEQ ID NO:2, AND SEQ ID NO:3, either in a single dsRNA construct, an admixture of constructs, or through concomitant administration of such constructs to a patient. SEQ ID NO:1, SEQ ID
- 15 NO:2, and SEQ ID NO:3 map to the HBV surface antigen genes. Due to the overlapping nature of the HBV mRNAs, the following mRNAs would be targeted by one of more of these sequences: Surface Ag (sAg) mRNAs, precore, core and polymerase mRNAs. However, since sAg mRNAs are the most abundant, it is more likely that these mRNAs will be targeted if
- 20 the gene-silencing machinery is saturable. It is possible, however, that all listed mRNAs will be targeted. Reduction of surface Ag is desirable for several reasons: a) surface Ag is needed for assembly of infectious virions; b) overexpression of Surface Ag during infection is thought to contribute to immune anergy that occurs during chronic infection; and c)
- 25 the expression of sAg in the livers of infected individuals (even in the absence of virus, i.e., from integrated sAg sequences into the host genome) induces hepatitis. Therefore, reduction of sAg is likely to decrease viral titers, overcome immune anergy and decrease/prevent hepatitis.
- 30 HBV SEQ ID NO:4 maps to the unique region of precore and core and will target these mRNAs specifically. Core protein is needed to make functional virions and so down regulation of this mRNA is predicted to

decrease viral titers. There should be no competition of these effector RNAs for surface, polymerase or X mRNAs.

HBV SEQ ID NO:5 through SEQ ID NO:8 map to the polymerase gene. Effector RNAs are predicted to target only precore/core and 5 polymerase transcripts. There should be no competition with sAg or X mRNAs. Polymerase is needed for the synthesis of viral genomes and therefore viral particle titer is expected to decrease if polymerase is decreased.

HBV SEQ ID NO:9 maps to the X gene. Due to the terminal 10 redundancy of all mRNAs, these effector RNAs have the potential to target all viral mRNAs. X protein has many ascribed (non proven) functions. However, the X gene is often found in integrated HBV sequences in individuals with active hepatitis and down-regulation of X gene expression is predicted to ameliorate disease.

15 In general, the more sequences from the different identified sequences that are used (e.g., from SEQ ID NO:1, SEQ ID NO:2, and/or SEQ ID NO:3, plus sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10), the less likely a virus will be able to generate viable escape mutants. Also, 20 the more different mRNAs that can be targeted, the more significant will be the drops in viral titer and disease amelioration.

Desirable combinations for multiepitope constructs, an admixture of constructs, or the concomitant administration of different dsRNA constructs include the following: Sequences from SEQ ID NO:1, SEQ ID 25 NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7; 30 Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9; Sequences

- from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:5; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus

sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9 and SEQ ID NO:10;

5 Sequences from SEQ ID NO:4 and SEQ ID NO:5; Sequences from SEQ ID NO:4 and SEQ ID NO:6; Sequences from SEQ ID NO:4 and SEQ ID NO:7; Sequences from SEQ ID NO:4 and SEQ ID NO:8; Sequences from SEQ ID NO:4 and SEQ ID NO:9; Sequences from SEQ ID NO:4 and SEQ ID NO:10; Sequences from SEQ ID NO:5 and SEQ ID NO:6; Sequences

10 from SEQ ID NO:5 and SEQ ID NO:7; Sequences from SEQ ID NO:5 and SEQ ID NO:8; Sequences from SEQ ID NO:5 and SEQ ID NO:9; Sequences from SEQ ID NO:5 and SEQ ID NO:10; Sequences from SEQ ID NO:6 and SEQ ID NO:7; Sequences from SEQ ID NO:6 and SEQ ID NO:8; Sequences from SEQ ID NO:6 and SEQ ID NO:9; Sequences from

15 SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:7 and SEQ ID NO:9; Sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences from SEQ ID NO:9 and SEQ ID NO:10; Sequences

20 from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:6; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6;

25 and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:7; and SEQ ID NO:9;

30 Sequences from SEQ ID NO:4, SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:8; and SEQ ID NO:10;

- Sequences from SEQ ID NO:4, SEQ ID NO:9; and SEQ ID NO:10;
Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:7;
Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:8;
Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:9;
- 5 Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:10;
Sequences from SEQ ID NO:5, SEQ ID NO:7; and SEQ ID NO:8;
Sequences from SEQ ID NO:5, SEQ ID NO:7; and SEQ ID NO:9;
Sequences from SEQ ID NO:5, SEQ ID NO:7; and SEQ ID NO:10;
Sequences from SEQ ID NO:5, SEQ ID NO:8; and SEQ ID NO:9;
- 10 Sequences from SEQ ID NO:5, SEQ ID NO:8; and SEQ ID NO:10;
Sequences from SEQ ID NO:5, SEQ ID NO:9; and SEQ ID NO:10;
Sequences from SEQ ID NO:6, SEQ ID NO:7; and SEQ ID NO:8;
Sequences from SEQ ID NO:6, SEQ ID NO:7; and SEQ ID NO:9;
Sequences from SEQ ID NO:6, SEQ ID NO:7; and SEQ ID NO:10;
- 15 Sequences from SEQ ID NO:6, SEQ ID NO:8; and SEQ ID NO:9;
Sequences from SEQ ID NO:6, SEQ ID NO:8; and SEQ ID NO:10;
Sequences from SEQ ID NO:6, SEQ ID NO:9; and SEQ ID NO:10;
Sequences from SEQ ID NO:7, SEQ ID NO:8; and SEQ ID NO:9;
Sequences from SEQ ID NO:7, SEQ ID NO:8; and SEQ ID NO:10;
- 20 Sequences from SEQ ID NO:7, SEQ ID NO:9; and SEQ ID NO:10;
Sequences from SEQ ID NO:8, SEQ ID NO:9; and SEQ ID NO:10;
Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:6; and SEQ ID
NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and
SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID
- 25 NO:6, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5;
SEQ ID NO:6; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ
ID NO:5; SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:4,
SEQ ID NO:5; SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID
NO:4, SEQ ID NO:5; SEQ ID NO:7; and SEQ ID NO:10; Sequences from
- 30 SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:8; and SEQ ID NO:9;
Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:8; and SEQ ID
NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:9; and

SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:4,
5 SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:7; SEQ ID NO:9; and SEQ ID NO:10;
Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and
10 SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:5,
SEQ ID NO:6; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ
15 ID NO:5, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:10;
Sequences from SEQ ID NO:5, SEQ ID NO:7; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and
SEQ ID NO:9; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID
20 NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7,
SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ
ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID
NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8;
Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID
25 NO:7, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5,
SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:10; Sequences from SEQ
ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:9;
Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID
NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5,
30 SEQ ID NO:6, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ
ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9;
Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID

NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID
5 NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID
10 NO:8, and SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ
15 ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID
20 ID NO:6, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID
25 NO:8, SEQ ID NO:9, and SEQ ID NO:10; and Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10.

In an another embodiment, combinations of sequitopes and longer sequences from within any of the aforementioned sequences (e.g., SEQ
30 ID NO:1 through SEQ ID NO:12) may be utilized either in a single dsRNA construct, an admixture of constructs, or through concomitant administration of such constructs to a patient.

As discussed elsewhere herein, a particularly preferred embodiment of the invention utilizes dsRNA expression constructs or vectors to achieve endogenous delivery of the dsRNAs of the invention, especially the multiple different sequences described above. These

5 dsRNAs may be provided e.g., on the same cistron of an expression construct such as a plasmid, on different cistrons of an expression construct, or on different expression constructs or plasmids, e.g., one or more plasmids and/or one or more vectors, including viral vectors. Such multiple different sequences may also be provided exogenously, in any

10 different mixture of one or more dsRNA structures, duplexes and/or hairpins, and/or in combination with one or more endogenously expressed dsRNA structures.

Desirable methods of administration of nucleic acids The DNA and/or RNA constructs of the invention may be administered to the host cell/tissue/organism as "naked" DNA, RNA, or DNA/RNA, formulated in a pharmaceutical vehicle without any transfection promoting agent. More efficient delivery may be achieved as known to those of skill in the art of DNA and RNA delivery, using e.g., such polynucleotide transfection facilitating agents known to those of skill in the art of RNA and/or DNA delivery. The following are exemplary agents: cationic amphiphiles including local anesthetics such as bupivacaine, cationic lipids, liposomes or lipidic particles, polycations such as polylysine, branched, three-dimensional polycations such as dendrimers, carbohydrates, detergents, or surfactants, including benzylammonium surfactants such as benzalkonium chloride. Non-exclusive examples of such facilitating agents or co-agents useful in this invention are described in U.S. Patent numbers 5,593,972; 5,703,055; 5,739,118; 5,837,533; 5,962,482; 6,127,170; 6,379,965; and 6,482,804; and International Patent Application No. PCT/US98/22841; the teaching of which is hereby incorporated by reference. U.S. Patents numbers 5,824,538; 5,643,771; and 5,877,159 (incorporated herein by reference) teach delivery of a composition other

than a polynucleotide composition, e.g., a transfected donor cell or a bacterium containing the dsRNA-encoding compositions of the invention.

In some embodiments, the dsRNA or dsRNA expression vector is complexed with one or more cationic lipids or cationic amphiphiles, such

5 as the compositions disclosed in US 4,897,355 (Eppstein *et al.*, filed October 29, 1987), US 5,264,618 (Felgner *et al.*, filed April 16, 1991) or US 5,459,127 (Felgner *et al.*, filed September 16, 1993). In other

10 embodiments, the dsRNA or dsRNA expression vector is complexed with a liposome/liposomal composition that includes a cationic lipid and optionally includes another component such as a neutral lipid (see, for example, US 5,279,833 (Rose), US 5,283,185 (Epand), and US 5,932,241).

Particularly desirable methods and compositions for delivery of the oligonucleotide compositions of the invention for pharmaceutical applications, including for targeted delivery to hepatocytes, are described in PCT/US03/14288, filed May 6, 2003, the teaching of which is incorporated herein by reference.

Transformation/transfection of the cell for research and other non-therapeutic purposes may occur through a variety of means including, but

20 not limited to, lipofection, DEAE-dextran-mediated transfection, microinjection, calcium phosphate precipitation, viral or retroviral delivery, electroporation, or biolistic transformation. The RNA or RNA expression vector (DNA) may be naked RNA or DNA or local anesthetic complexed RNA or DNA (See U.S. Patent Nos. 6,217,900 and 6,383,512, "Vesicular

25 Complexes and Methods of Making and Using the Same, Pachuk *et al.*, *supra*).

Another desirable delivery technology for the dsRNAs or dsRNA expression constructs of the invention for pharmaceutical applications is the self-assembling CyclosertTM two-component nucleic acid delivery system, based on cyclodextrin-containing polycations, which are available from Insert Therapeutics, Pasadena, CA. (See Bioconjug Chem 2003 May-Jun; 14 (3): 672-8; Popielarski *et al.*; "Structural effects of

carbohydrate-containing polycations on gene delivery. 3. Cyclodextrin type and functionalization"; as well as Bioconjug Chem 2003 Jan-Feb;14 (1):247-54 and 255-61.) The first component is a linear, cyclodextrin-containing polycationic polymer, that when mixed with DNA, binds to the phosphate "backbone" of the nucleic acid, condensing the DNA and self assembling into uniform, colloidal nanoparticles that protect the DNA from nuclease degradation in serum. A second component is a surface modifying agent with a terminal adamantine-PEG molecule, that when combined with the cyclodextrin polymer forms an inclusion complex with

5 surface cyclodextrins and prevents aggregation, enhances stability and enables systemic administration. In addition, targeting ligands to cell surface receptors may be attached to the modifier providing for targeted delivery of DNA directly to target cells of interest. Since hepatocytes are susceptible to HBV and HCV infection, utilizing this method to target

10 delivery of the dsRNA expression constructs of the invention to liver cells is considered especially advantageous. E.g., the asialoglycoprotein receptor (ASGP-R) on mammalian hepatocytes may be targeted by use of synthetic ligands with galactosylated or lactosylated residues, such as galactosylated polymers.

15

20 In general, targeting for selective delivery of the dsRNA constructs of the invention to hepatocytes is preferred. Targeting to hepatocytes may be achieved by coupling to ligands for hepatocyte-specific receptors. For example, asialo-orosomucoid, (poly)L-lysine-asialo-orosomucoid, or any other ligands of the hepatic asialoglycoprotein receptor (Spiess,

25 Biochemistry 29(43):10009-10018, 1990; Wu et al., J. Biol. Chem. 267(18):12436-12439, 1992; Wu et al., Biotherapy 3:87-95, 1991).

Similarly, the oligonucleotides may be targeted to hepatocytes by being conjugated to monoclonal antibodies that specifically bind to hepatocyte-specific receptors. Oligonucleotides may also be targeted to hepatocytes

30 using specific vectors, as described below.

Particularly preferred compositions for delivery of dsRNAs or dsRNA expression constructs of the invention are the multifunctional

compositions as described in PCT/US03/14288, filed May 6, 2003, which may include trilactosyl spermine as a ligand for targeting to the ASG Receptor of hepatocytes. Trilactosyl cholestryl spermine co-complexes with the oligonucleotides of the invention may be prepared and used as 5 described to transfect hepatocytes *in vivo*.

The dsRNA oligonucleotides of the invention may be provided exogenously to a target hepatocyte. Alternatively, a dsRNA may be produced within the target cell by transcription of a nucleic acid molecule comprising a promoter sequence operably linked to a sequence encoding 10 the dsRNA. In this method, the nucleic acid molecule is contained within a non-replicating linear or circular DNA or RNA molecule, or is contained within an autonomously replicating plasmid or viral vector, or is integrated into the host genome. Any vector that can transfect a hepatocyte may be used in the methods of the invention. Preferred vectors are viral vectors, 15 including those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses (see, e.g., WO89/07136; Rosenberg et al., N. Eng. J. Med. 323(9):570-578, 1990), adenovirus (see, e.g., Morsey et al., J. Cell. Biochem., Supp. 17E, 1993; Graham et al., in Murray, ed., Methods in Molecular Biology: Gene Transfer and Expression Protocols. 20 Vol. 7, Clifton, N.J.: the Human Press 1991: 109-128), adeno-associated virus (Kotin et al., Proc. Natl. Acad. Sci. USA 87:2211-2215, 1990), replication defective herpes simplex viruses (HSV; Lu et al., Abstract, page 66, Abstracts of the Meeting on Gene Therapy, Sep. 22-26, 1992, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), and any modified 25 versions of these vectors. Methods for constructing expression vectors are well known in the art (see, e.g., Molecular Cloning: A Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, N.Y., 1989).

Appropriate regulatory sequences can be inserted into the vectors 30 of the invention using methods known to those skilled in the art, for example, by homologous recombination (Graham et al., J. Gen. Virol. 36:59-72, 1977), or other appropriate methods (Molecular Cloning: A

Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, N.Y., 1989).

Promoters Promoters are inserted into the vectors so that they are operably linked, typically but not invariably, 5' to the nucleic acid sequence encoding the dsRNA oligonucleotide. Any promoter that is capable of directing initiation of transcription in a eukaryotic cell may be used in the invention. For example, non-tissue-specific promoters, such as the cytomegalovirus (DeBernardi et al., Proc. Natl. Acad. Sci. USA 88:9257-9261, 1991, and references therein), mouse metallothioneine I gene (Hammer, et al., J. Mol. Appl. Gen. 1:273-288, 1982), HSV thymidine kinase (McKnight, Cell 31:355-365 1982), and SV40 early (Benoist et al., Nature 290:304-310, 1981) promoters may be used. Non-tissue-specific promoters may be used in the invention, as expression of HBV and/or HCV dsRNA in non-liver cells directed by the non-tissue-specific promoters should be harmless to the non-liver cells, because of the specificity of the HBV and HCV dsRNAs of the invention for viral sequences. However, preferred promoters for use in the invention are hepatocyte-specific promoters, the use of which ensures that the RNAs are expressed primarily in hepatocytes. Preferred hepatocyte-specific promoters include, but are not limited to the albumin, alpha-fetoprotein, alpha-1-antitrypsin, retinol-binding protein, and asialoglycoprotein receptor promoters. Viral promoters and enhancers, such as those from cytomegalovirus, herpes simplex viruses (types I and II), hepatitis viruses (A, B, and C), and Rous sarcoma virus (RSV; Fang et al., Hepatology 10:781-787, 1989), may also be used in the invention.

dsRNA expression vectors may include promoters for RNA polymerase I, RNA polymerase II including but not limited to HCMV, SCMV, MCMV, RSV, EF2a, TK and other HSV promoters such as ICP6, ICP4 and ICP0 promoters, HBV pregenomic promoter, RNA pol III promoter including but not limited to U6 and tRNA promoters, mitochondrial light and heavy strand promoters. Desirably, the dsRNA expression vector comprises at least one RNA polymerase II promoter, for

example, a human CMV-immediate early promoter (HCMV-IE) or a simian CMV (SCMV) promoter, at least one RNA polymerase I promoter, or at least one RNA polymerase III promoter. The promoter may also be a T7 promoter, in which case, the cell further comprises T7 RNA polymerase.

5 Alternatively, the promoter may be an SP6 promoter, in which case, the cell further comprises SP6 RNA polymerase. The promoter may also be one convergent T7 promoter and one convergent SP6 RNA promoter. A cell may be made to contain T7 or SP6 polymerase by transforming the cell with a T7 polymerase or an SP6 polymerase expression plasmid,

10 respectively. In some embodiments, a T7 promoter or a RNA polymerase III promoter is operably linked to a nucleic acid that encodes a short dsRNA (e.g., a dsRNA that is less than 200, 150, 100, 75, 50, or 25 basepairs in length). In other embodiments, the promoter is a mitochondrial promoter that allows cytoplasmic transcription of the nucleic acid in the vector (see, for example, the mitochondrial promoters described in WO 00/63364, filed April 19, 2000, and in WO/US2002/00543, filed 9-Jan-2001). Alternatively, the promoter is an inducible promoter, such as a *lac* (Cronin et al. *Genes & Development* 15: 1506-1517, 2001), *ara* (Khlebnikov et al., *J Bacteriol.* 2000

15 Dec;182(24):7029-34), ecdysone (Rheogene website), RU48 (mefepristone) (corticosteroid antagonist) (Wang XJ, Liefer KM, Tsai S, O'Malley BW, Roop DR, *Proc Natl Acad Sci U S A.* 1999 Jul 20;96(15):8483-8), or *tet* promoter (Rendal et al., *Hum Gene Ther.* 2002;13(2):335-42 and Larnartina et al., *Hum Gene Ther.* 2002;13(2):199-

20 210) or a promoter disclosed in WO 00/63364, filed April 19, 2000. Also useful in the methods and compositions of the invention are the structural and chimeric promoters taught in USSN 60/464,434, filed 22-Apr-2003. See also the promoter systems taught in Pachuk, C., and Satishchandran, C., "Multiple-Compartment Eukaryotic Expression Systems," U.S.

25 30 Provisional Application No. 60/497,304, filed 22-Aug-2003, which are considered particularly desirable in the methods and compositions of the invention.

Liver specific promoters useful in dsRNA expression constructs of the invention include the albumin promoter, the alpha-fetoprotein promoter (especially in liver cancer cells), the alpha-1-antitrypsin promoter, hepatitis B promoters, e.g., hepatitis B promoters including promoters for the 5 antigen genes, including core, e antigen, polymerase and X protein.

- T7 Promoter/T7 Polymerase Expression Systems A desirable method of the invention utilizes a T7 dsRNA expression system to achieve cytoplasmic expression of dsRNA, (e.g., long or short dsRNA molecules) in vertebrate cells (e.g., mammalian cells). The T7 expression system 10 utilizes the T7 promoter to express the desired dsRNA. Transcription is driven by the T7 RNA polymerase, which can be provided on a second plasmid or on the same plasmid. Bacteriophage T7 RNA polymerase (T7 Pol) is the product of T7 gene 1, which can recognize its responsive promoter sequence specifically and exhibit a high transcriptase activity.
- 15 The complete sequence of the T7 genome, with detailed information about the different regions of the bacteriophage, including promoter sequences, is disclosed in Dunn & Studier, 1983, J. Mol. Biol. 166(4), 477-535 (see also NCBI 'Genome' database, Accession No. NC 001604). The T7 promoter cannot be utilised by any other RNA polymerase than the 20 polymerase of bacteriophage T7, which shows a stringent specificity for the promoter (Chamberlin et al., 1970, Nature 228:227-231). When utilizing the T7 expression system for expressing dsRNAs, for example, a first plasmid construct that expresses both a sense and antisense strand under the control of converging T7 promoters and a second plasmid 25 construct that expresses the T7 RNA polymerase under the control of an RSV promoter can be used. Both the dsRNA and the T7 RNA polymerase could advantageously be expressed from a single bicistronic plasmid construct, particularly when the dsRNA is formed from a single RNA strand with inverted repeats or regions of self-complementarity that enable the 30 strand to assume a stem-loop or hairpin structure with an at least partially double stranded region. Individual sense and antisense strands which self assemble to form a dsRNA can be synthesized by a single plasmid

construct using, e.g., converging promoters such as bacteriophage T7 promoters placed respectively at the 5' and 3' ends of the complementary strands of a selected sequence to be transcribed. See also, e.g., the teaching of WO 0063364, with respect to T7 dsRNA expression systems,
5 as well as USSN 60/399,998P, filed 31-Jul-2002 and USSN 60/419,532, filed 18-Oct-2002.

Therapeutic Compositions of the Invention The dsRNAs of the invention, and the recombinant vectors containing nucleic acid sequences encoding them, may be used in therapeutic compositions for preventing or
10 treating HCV and/or HBV infection. The therapeutic compositions of the invention may be used alone or in admixture, or in chemical combination, with one or more materials, including other antiviral agents. Currently, lamivudine, adefovir dipivoxil, and interferon alpha have been approved for treatment of HBV, and it is anticipated that the compositions of the
15 invention may be used in combination with these and other drugs that are active against HBV, including emtricitabine (FTC) and entecavir. Since dsRNAs against HBV and/or HCV act through a novel mechanism (dsRNA-mediated gene silencing/RNAi), combination therapy of the agents of the invention and other antivirals is expected to significantly
20 increase the efficacy of therapy while substantially reducing the development of drug resistance, e.g., the development of lamivudine resistance, a problem of major concern with long term lamivudine therapy. Currently, interferon and ribavirin are licensed for treatment of HCV, and as for HBV, it is anticipated that the compositions of the invention may be
25 used in combination with these and other drugs that are active against HCV. Specific dosage regimens involving therapy with such multiple agents can be determined through routine experimentation by those of ordinary skill in the art of clinical medicine.

Formulations will desirably include materials that increase the
30 biological stability of the oligonucleotides or the recombinant vectors, or materials that increase the ability of the therapeutic compositions to penetrate hepatocytes selectively. The therapeutic compositions of the

invention may be administered in pharmaceutically acceptable carriers (e.g., physiological saline), which are selected on the basis of the mode and route of administration, and standard pharmaceutical practice. One having ordinary skill in the art can readily formulate a pharmaceutical composition that comprises an oligonucleotide or a gene construct. In some cases, an isotonic formulation is used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vasoconstriction agent is added to the formulation. The pharmaceutical preparations according to the present invention are provided sterile and pyrogen free. Suitable pharmaceutical carriers, as well as pharmaceutical necessities for use in pharmaceutical formulations, are described in Remington: The Science and Practice of Pharmacy (formerly Remington's Pharmaceutical Sciences), Mack Publishing Co., a standard reference text in this field, and in the USP/NF.

Routes of administration include, but are not limited to, intramuscular, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraocularly and oral as well as transdermally or by inhalation or suppository. Preferred routes of administration include intravenous, intramuscular, oral, intraperitoneal, intradermal, intraarterial and subcutaneous injection. dsRNAs or dsRNA expression constructs may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microprojectile bombardment gene guns". Alternatively, the dsRNA and/or dsRNA expression construct may be introduced by various means into cells that are removed from the individual. Such means include, for example, *ex vivo* transfection, electroporation, microinjection and microprojectile bombardment. After the gene construct is taken up by the cells, they are reimplanted into the individual. It is contemplated that otherwise non-immunogenic cells that have gene constructs incorporated therein can be implanted into the

individual even if the host cells were originally taken from another individual.

- In HBV infected individuals it is anticipated that the dsRNA compositions of the invention may be useful as a pre-treatment in conjunction with therapeutic vaccination protocols designed to boost immunity against the virus. It is also anticipated that the dsRNA compositions of the invention may be useful for prophylaxis in a regimen of periodic administrations to individuals who because of occupational or other potential for exposure are considered at high risk of exposure to HBV and/or HCV, e.g., fire, emergency, and health care personnel. Such an effective prophylactic regime may include administration of a composition that provides an HBV and/or HCV dsRNA of the invention, e.g., weekly, biweekly, monthly, bimonthly, every three months, every four months, semi-yearly, or yearly, as can be determined through routine experimentation by those of skill in the art of clinical medicine. The ability of a dsRNA expression vector such as a plasmid or viral vector to express the dsRNAs of the invention over a relatively prolonged period of time, expected to be in the range of weeks to months, is considered to be advantageous for this and other applications.
- 20 Dosage of dsRNAs** For administration of dsRNA (e.g., a short dsRNA to inhibit toxicity or a short or long dsRNA to silence a gene) to an animal, typically between 10 mg to 100 mg, 1 mg to 10 mg, 500 µg to 1 mg, or 5 µg to 500 µg dsRNA is administered to a 90-150 pound person/animal (in order of increasing preference). For administration of a vector encoding dsRNA (e.g., a short dsRNA to inhibit toxicity or a short or long dsRNA to silence a gene) to an animal, typically between 100 mg to 300 mg, 10 mg to 100 mg, 1 mg to 10 mg, 500 µg to 1 mg, or 50 µg to 500 µg dsRNA expression vector or construct is administered to a 90-150 pound person/animal (in order of increasing preference). The dose may be adjusted based on the weight of the animal. In some embodiments, about 1 to 10 mg/kg or about 2 to 2.5 mg/kg is administered. Other doses

may also be used, as determined through routine experimentation by those of skill in the art of clinical medicine.

For administration in an intact animal, typically between 10 ng and 50 µg, between 50 ng and 100 ng, or between 100 ng and 5 µg of dsRNA or DNA encoding a dsRNA is used. In desirable embodiments, approximately 10 µg of a DNA or 5 µg of dsRNA is administered to the animal. With respect to the methods of the invention, it is not intended that the administration of dsRNA or DNA encoding dsRNA to cells or animals be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration sufficient to provide a dose adequate to inhibit gene expression, prevent a disease, or treat a disease.

If desired, short dsRNA is delivered before, during, or after the exogenous delivery of dsRNA (e.g., a longer dsRNA) that might otherwise be expected to induce cytotoxicity. See the teaching of USSN 10/425,006, filed 28-Apr-2003, "Methods of Silencing Genes Without Inducing Toxicity", Pachuk.

Applicants specifically incorporate the entire content of all cited references in this disclosure. Further, when an amount, concentration, or other value or parameter is given as either a range, preferred range, or a list of upper preferable values and lower preferable values, this is to be understood as specifically disclosing all ranges formed from any pair of any upper range limit or preferred value and any lower range limit or preferred value, regardless of whether ranges are separately disclosed.

Where a range of numerical values is recited herein, unless otherwise stated, the range is intended to include the endpoints thereof, and all integers and fractions within the range. It is not intended that the scope of the invention be limited to the specific values recited when defining a range.

EXAMPLES

The following Examples are provided as illustrative only. All references mentioned within this disclosure are specifically incorporated herein by reference in their entirety.

5

EXAMPLE 1

Silencing HBV replication and expression in a replication competent cell culture model

Brief description of cell culture model: A human liver-derived cell
10 line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession V01460. Following internalization
15 into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic
20 RNA, and assembly and secretion of virions and HBsAg (Hepatitis B Surface Antigen) particles into the media of transfected cells. This transfection model reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression and replication.

25 Using this model, cells were co-transfected with the infectious molecular clone of HBV and various eiRNA constructs. The cells were then monitored for loss of HBV expression and replication as described below. Details on the vector and encoded RNAs used in this experiment are provided at the end of this example.

30

Experiment 1:

The following is an example of an experiment that was performed using eiRNA vectors encoding sequences derived from GenBank accession number V01460. HBV sequences in these described eiRNA 5 vectors were highly conserved sequences identified as described elsewhere herein, which also exhibited activity as siRNAs (See, Pachuk, C., "Methods and Constructs for Evaluation of RNAi targets and Effector Molecules," PCT/US2004/005065, filed 25-Feb-2004). The particular eiRNA backbone vector used for this experiment was a proprietary vector 10 containing a U6 promoter to drive expression of the encoded RNAs. Each vector encoded only one short hairpin RNA (shRNA). The shRNA coding sequence was followed by an RNA pol III termination sequence. Sequences of the U6 promoter, RNA pol III termination signal, and encoded shRNAs are all shown at the end of the example. Similar vectors 15 containing U6 promoters and RNA pol III termination signals are commercially available such as the "siLentGene-2 Cloning Systems" vector from Promega, Inc., Madison, Wis. One of ordinary skill in the art can also create them according to the information provided herein. It is expected that similar results would also be obtained using other 20 expression and promoter systems especially those vectors with RNA pol III promoters that are not U6, for example H1 promoters or 7SK promoters.

Experimental Procedure: Transfection.

Huh7 cells cultured in RPMI-1640 media were seeded into six-well 25 plates at a density of 3×10^5 cells/well. All transfections were performed the day after cell seeding using LipofectamineTM (InVitrogen, Carlsbad, Cal.) according to the manufacturer's directions. In this experiment, cells were transfected with 500 ng of the infectious HBV plasmid ayw subtype ("pHBV2") (GenBank Accession # V01460) and 500 ng, 300ng, 250ng, 30 120 ng, 100 ng, 50ng, or 10 ng of an eiRNA construct. DNA was held constant/transfection at 2.5 μ g by including an inert plasmid DNA, pGL3-Basic (Promega, Madison Wis.) in amounts that brought the total DNA in

the transfection to 2.5 µg. For example, in transfections receiving 500 ng of HBV DNA and 500 ng of an eiRNA construct, 1.5 µg pGL3 was added to the transfection. Prior to transfection, media was removed from the cells and the cells washed with Opti-MEM® (InVitrogen Life Technologies, Carlsbad, Cal.). 800 µl of Opti-MEM® was then added to each well of cells followed by the addition of the transfection mix. Seventeen to nineteen hours post-transfection, the transfection mix and Opti-MEM® were removed from cells and replaced with 2 mL culture media/well. At 3, 6, and 10 days after transfection, the media was removed from cells and stored at -70 °C. The media was replaced with 2 mL of fresh culture media on days 3 and 6. All transfections were carried out in duplicate. Two sets of control transfections were also performed: HBV DNA alone (500 ng HBV DNA plus 2 µg pGL3) and HBV DNA with a control eiRNA construct (500 ng HBV DNA, 1 µg control eiRNA construct, and 1.0 µg pGL3 DNA).

Monitoring cells for loss of HBV expression.

Following transfection, cells were monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion. Cells were monitored by assaying the media of transfected cells (and a media control) at days 3, 6, and 10 post-transfection. The Auszyme® ELISA, commercially available from Abbott Labs (Abbott Park, Ill.), was used to detect surface Ag (sAg) according to the manufacturer's instructions. sAg was measured since surface Ag is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected infectious HBV clone and from HBVcccDNA produced during infection *in vivo*. Since surface Ag synthesis can continue with deleterious effects in the absence of HBV replication, it is important to down-regulate not only viral replication but also replication-independent synthesis of sAg.

Results:

Cells transfected with the HBV-specific eiRNA constructs described at the end of this example all induced a decrease in sAg levels relative to the controls. The level inhibition is shown in the accompanying FIG. 2-8 corresponding to data found in Tables 2-8. Note that the sequences identified as 788-808 and 807-827 only lowered surface Ag levels by 30% and 50% respectively at 500 ng doses. These are the only two eiRNAs that do not target the sAg mRNA; instead they target the 3.1 Kb HBV mRNAs and therefore reduce sAg levels indirectly. The 30% to 50% reduction in sAg observed when these other HBV RNAs are targeted is considered a strong indication that these eiRNA constructs are efficacious.

HBV-specific eiRNAs used in this experiment

The eiRNA vectors encode the HBV sequences listed in Table 1. The sequences are shown as well as their map coordinates on GenBank accession number V01460. At the rightmost part of the table is the SEQ ID NO that these sequences map within. The sequence of the encoded RNA is 5'GGTCGAC (a sequence that is *per se* unimportant, but is derived from the polylinker sequence of the particular vector used) followed by a first sense or antisense HBV sequence followed by the loop sequence (underlined in Table 1) followed by a second HBV sequence, which is the complement to the first HBV sequence. Note that the loop structure does not need to be a fixed sequence or length, and we have used several loop sequences with no significant impact on the functioning of the eiRNA construct. The second HBV sequence is followed by a string of T residues, e.g., 1, 2, 3, or more Ts, that function as the termination signal for RNA pol III.

Table 1

<u>HBV-AYW</u> coordinates*	<u>SEQ ID</u> <u>NO</u>	sequence (sense stem - loop - antisense stem)	<u>Maps within</u> <u>SEQ ID NO</u>
788-808	14	CGTCTGGGAGGGAGTTAGAGAACTTAACCTCCCTCGCCCTCGCAAGACG	5, 6, 7, or 8
807-827	15	TTCTTCTTCTAGGGGACCTGCAGAGAACCTTGAGGTCCCCTAGAAAGAAGAA	5, 6, 7, or 8
1291-1311	16	AAGCCACCCAAAGGCCACAGCTTAGAGAACCTTAAGGCTGTGCCCTGGCTT	4
1299-1319	17	CAAGGCACAGCTGGAGGGCTTAGAGAACCTTAAGCCTCCAAGCTGTGCCCTTG	4
1737-1757	18	GGATTTCAGCGCCGGACGGGACGGAGAGAACCTTCGTCCCCGTCGGGCTGAATCC	10
1907-1927	19	TICCGCAGTATGGATCGGCAGAGAGAACCTTCTGCCGATCCATACTGGGAA	3
1912-1932	20	CAGTATGGATCGGCAGAGGAGAGAGAACCTTGGCCATCAGGGCATGCGTGGAA	3
1943-1963	21	TCCACGGCATGGCCTGATGGCAGAGAACCTGGCCAGAGAACCTTGGGAACTTGCAAGTGTGGCA	3
1991-2011	22	TGCCTCAGCAAACACCTGGCAAGAGAACCTTGGCAAGTGTGGTGTGACGCA	3
2791-2811	23	AAAACGGCCGAGACACATCCAAAGAGAACCTTGGATGTGTGGGTGTTT	2
2791-2811mut	24	AAAACACCACACGGCATCCAAAGAGAACCTTGGATGCCTGTGGTGTGTTT	2
2912-2932	25	TTGAGAGAAGTCCACCAACGGAGAGAACCTTCGTGGGACTTCTCAA	1
2919-2939	26	AAGTCCACCAACGGAGTCTAGACAGAGAACCTGTCTAGACTCGTGGGACTT	1

*nucleotide coordinates refer to Genbank accession number V01460.

A diagram of the transcribed RNA structure is shown in FIG. 9.

SEQ ID NO:13 is the nucleotide sequence of U6 promoter.

Nucleotide sequence of RNA pol III terminator: 5' – TTTTT – 3'.

Tables and Graphs.

HBsAg was measured as described above and plotted in FIG. 2-8 corresponding to the data in Tables 2-8. The amount of eiRNA construct is shown in parentheses following the name of the eiRNA construct and is in µg amounts. For example, 2791(0.5) means that 0.5 µg or 500 ng of eiRNA construct 2791-2811 (see Table 1) was used in the transfection. The percent inhibition relative to the control is also shown in the tables below and it is specific for the day 10 measurement. Note that the 4th set of data in this example in which 1299 was evaluated at 500 ng has only two timepoints, days 3 and 6, because the evaluation was not carried out at day 10. The percent inhibition for this experiment was shown for day 6 data. Data is shown as raw OD data collected as described by the manufacturer of the Auszyme ELISA assay kit used to measure sAg. Not shown are the 50 ng data for 2791-2811 and the 10ng data for 1907-1927. Each of these doses inhibited HBsAg expression by about 50% relative to the control.

Table 2

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.339	1.88	3.268	-----
2791(0.5)	0.101	0.263	0.333	89.8

Table 3

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	1.169	4.445	10.18	-----
2791(0.5)	0.442	0.743	1.3	87.2
2791Mut(0.5)	1.136	4.305	10.595	-----

Table 4

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.375	1.952	4.005	-----
2791mut(1)	0.421	1.847	4.753	-----
HCV(1)	0.445	1.805	3.933	-----
788(0.5)	0.255	1.195	2.778	30.6
807(0.5)	0.254	1.326	2.015	49.7
1907(0.25)	0.052	0.113	0.365	90.9
1912(0.25)	0.138	0.208	0.517	87.1
1943(0.25)	0.099	0.233	0.506	87.4
1991(0.25)	0.075	0.152	0.291	92.7
2912(0.25)	0.095	0.183	0.331	91.7

Table 5

	Day 3	Day 6	% Inhibition relative to control
pHBV2	0.474	1.513	-----
1299(0.5)	0.439	0.699	53.8

Table 6

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.33	1.617	2.88	-----
2791(0.3)	0.103	0.192	0.349	87.9
1737(0.3)	0.051	0.094	0.232	91.9
1291(0.12)	0.239	0.587	1.195	58.5
1907(0.12)	0.043	0.086	0.356	87.6
2919(0.12)	0.218	0.565	1.09	62.2

Table 7

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.741	2.53	5.383	-----
2791(0.3)	0.223	0.256	0.458	91.5
1737(0.1)	0.212	0.351	0.549	89.8
1907(0.1)	0.067	0.149	0.468	91.3
1991(0.1)	0.067	0.16	0.345	93.6

Table 8

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.864	4.414	8.344	-----
1907(0.05)	0.17	0.538	1.396	83.3
2919(0.1)	0.368	1.044	1.908	77.1
1291(0.2)	0.573	1.654	1.896	77.3

Experiment 2:

Background: The same cell culture model was used to evaluate the additive effects of adding two eiRNA constructs. In this experiment 2791-2811 and 2919-2939 were evaluated. They were evaluated separately at two doses: 10 ng and 25 ng, and in combination at 10 ng (5 ng of 2791-2811 plus 5 ng of 2919-2939) and at 25 ng (12.5 ng 2791-2811 plus 12.5ng 2919-2939). An additive effect is observed, for example, when half the inhibition seen with 25 ng 2791-2811 plus half the inhibition seen with 25 ng 2919-2939 is about equal to the inhibition seen of the 25 ng combination dose. This is important because while one may not be gaining inhibition over the use of a single eiRNA construct at the 25 ng dose, the use of two or more eiRNA sequences is very important in preventing the generation of viral escape mutants.

Experimental Procedure: Transfection.

Huh7 cells were seeded into six-well plates at a density of 3×10^5 cells/well. All transfections were performed the day after cell seeding using Lipofectamine™ (InVitrogen) according to the manufacturer's

directions. In this experiment, cells were transfected with 500 ng of the infectious HBV plasmid ayw subtype (GenBank Accession # V01460) and 25 ng or 10 ng of two separate eiRNA constructs or a combination of these two eiRNA constructs at a total of 25 ng or 10 ng. DNA was held constant/transfection at 2.5 µg by including an inert plasmid DNA, pGL3, in amounts that brought the total DNA in the transfection to 2.5 µg. For example, in transfections receiving 500 ng of HBV DNA and 10 ng of an eiRNA construct, then 1.99 µg pLUC was added to the transfection. Prior to transfection, media was removed from the cells and the cells washed with Opti-MEM® (InVitrogen Life Technologies). 800 µl of Opti-MEM® was then added to each well of cells followed by the addition of the transfection mix. Seventeen to nineteen hours post-transfection, the transfection mix and Opti-MEM® was removed from cells and replaced with 2 mL culture media/well. At 4, 8, and 11 days after transfection, the media was removed from cells and stored at -70 °C. The media was replaced with 2 mL of fresh culture media on days 4 and 8. All transfections were carried out in duplicate. Two sets of control transfections were also performed: HBV DNA alone (500 ng HBV DNA plus 2 µg pGL3), and HBV DNA with a control eiRNA construct (500 ng HBV DNA, 500 ng control eiRNA construct and 1.5µg pGL3. DNA).

Results:

Results are shown in Table 9, and the corresponding graph found in **FIG. 10.** Combining 2791-2811 and 2919-2939 showed at least equal effects to administration of 2791-2811 or 2919-2939 alone. It is expected that similar advantages will be seen by combining two or more dsRNAs directed to different HBV sequences from the same and/or different HBV genes.

Table 9

	Day 4	Day 8
pHBV ₂	3.74	15.03
2791 @ 25 ng	2.49	9.63
2919 @ 25 ng	2.55	10.07
2791 + 2919 @ 25 ng	2.73	10.91

Experiments 3 and 4**Silencing of HBV in a mouse model.**

Summary: Two of the eiRNA vectors described in confirmatory experiment 1 were assessed for their ability to silence an HBV replicon in a mouse model. These vectors were the 2791-2811 and the 1907-1927 vectors. Both vectors were found to silence HBV in the mouse model to a similar extent as they silenced in the cell culture model. The ability to silence this HBV replicon in mice by other therapeutics has been demonstrated to be a predictor of human efficacy [4].

Animal Model Background:

Chimpanzees represent the only animal model in which to study human HBV infectivity. A mouse model is available, however, in which HBV expression and replication occur. This model has been invaluable for the evaluation of anti-HBV therapeutic agents not only targeted against viral replication but also against RT-independent expression of antigen. In this model, replication competent HBV is expressed transiently from episomal HBV DNA. This model is created by introducing replication competent HBV DNA into mouse liver by hydrodynamic delivery [1].

The aim of the following experiment was to test two of the vectors encoding HBV-specific sequences evaluated in Experiment 1 for efficacy in a mouse model even though there were not expected to be HBV-sequence-related efficiency differences between the cell culture and mouse models. This experiment utilized hydrodynamic delivery as a method to co-deliver replication competent HBVayw plasmid (Example 1,

confirmatory experiment 1) with an effector HBV-specific eiRNA expression vector. Hydrodynamic delivery is ideal for these first studies because it results in efficient delivery of nucleic acid to the liver [5].

Experiments.

Hydrodynamic Delivery Studies: Experiment 3.

All animals were hydrodynamically injected with 7.5 µg infectious HBVayw plasmid (described in confirmatory Example 1). Following internalization into hepatocytes and nuclear localization, transcription of HBVayw plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication [1]. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the sera of injected animals. Experimental animals were co-injected with 10 µg 2791-2811. A second group of control animals were injected with 10 µg of an irrelevant eiRNA construct. All animals were also co-injected with 2.5 µg of a GFP reporter plasmid (Clontech, Palo Alto, Cal.). Expression of GFP mRNA in the livers of injected mice served as a control to normalize results against the mouse model transfection efficiency. Total DNA injected in animals was kept at a constant 20 µg by including pGL3, an inert filler DNA (Promega, Madison, Wis.). All DNA was formulated and injected according to the methods described in Yang et al. [1]. There were 5 animals per group. The DNAs and amounts of DNA injected per animal are shown in Table 10.

Table 10

Group	HBV DNA	GFP DNA	eiRNA	pGL3
1	7.5 µg	2.5 µg	10 µg 2791	0 µg
2	7.5 µg	2.5 µg	10 µg control	0 µg
3	7.5 µg	2.5 µg	0 µg	10 µg

Timepoints of analysis were selected based on published results from Dr. Chisari's laboratory [1], which detail the kinetics of HBVayw plasmid replication in mice following hydrodynamic delivery. Serum was assayed for the presence of HBsAg on days 1, 2, 3, and 4 post-injection. Assays were performed as described for the cell culture model of HBV replication. The presence of HBV RNA in liver samples was ascertained by Northern blot analysis on day 2 following injection using procedures developed in Dr. Chisari's laboratory [1] and normalized to endogenous GAPDH RNA levels and GFP mRNA levels using conventional techniques, or a quantitative RT-PCR assay for HBV RNAs containing sAg coding sequences using standard techniques. RT-PCR is more quantitative than Northern Blot analysis and has a larger dynamic window than does Northern Blot analysis.

Downregulation of both HBV RNA by Northern Blot analysis and HBsAg were seen in mice injected with 2791-2811. See FIG. 11. Also not shown, quantitative RT-PCR demonstrated the presence of 867 HBV RNA molecules in the livers of control mice and 57 molecules of HBV RNA in 2791-2811 treated mice, a 15-fold downregulation.

Table 11

Group	Mouse	3.5 kb	2.1 kb	GFP	HBV total	HBV/GFP	Group Average	Std. Dev
10 µg 2791	1	182360	1440614	4044344	1622974	4.0	3.6	0.76
	2	392294	3161703	9954889	3553997	3.6		
	3	268673	3114347	15317275	3383020	2.2		
	4	394799	3909096	16806285	4303895	2.6		
	5	362182	4439430	18306755	4801612	2.6		
HBV only	21	2412562	8720964	3860082	11133526	28.8	16.6	7.40
	22	2170741	7958388	6110744	10129129	16.6		
	23	2713213	12060855	9633404	14774068	15.3	17.4	7.40
	24	1924373	7243024	11042915	9167397	8.3		
	25	1464641	5726217	3968243	7190858	18.1		

Table 12

NUC5_HBsAg		HBsAg (ng/ml)			
		d1	d2	d3	d4
HBV	2	2810	6793	8422	8517
	3	2344	8332	8089	8743
	4	1684	8788	9064	8876
	5	2318	9378	8597	8480
	29	1066	5038	5153	5925
	grp ave=>	2044	7666	7865	8108
Std Dev=>		678	1754	1556	1231
eiHCV	6	2554	8048	9233	8870
	9	2267	8420	9535	8338
	10	1704	8258	8761	7840
	30	1362	4171	5406	4920
	grp ave=>	1972	7224	8234	7492
	Std Dev=>	538	2041	1912	1765
2791	11	1262	2823	2276	2080
	12	1222	2549	2858	1593
	14	1056	1933	1143	792
	15	1275	8320	1920	2068
	27	779	4771	3782	1252
	grp ave=>	1119	4079	2396	1557
Std Dev=>		209	2598	993	551

Hydrodynamic Delivery Studies: Experiment 4.

5 This experiment was similar to the Experiment 3 of Example 1 except that two eiRNA constructs were evaluated: 2791-2811 and 1907-1927. In this experiment, HBsAg was measured on days 1 and 4 using the assay already described herein.

Table 13

NUC6_HBsAg		HBsAg (ng/ml)	
		d1	d4
HBV	2	6147 ^	36,953 ^
	3	6234 ^	42,542 ^
	4	4658	33,061 ^
	5	5077 ^	29,389 ^
	grp ave=>	5529	35486
	Std Dev=>	784	5627
eiHCV	6	1901	11,236
	7	6286 ^	29,637 ^
	8	1023	6,345
	grp ave=>	3070	15739
	Std Dev=>	2820	12282
2791	11	3966	5009
	13	4705	7347 ^
	14	2289	4538
	15	2427	4217
	grp ave=>	3347	5278
	Std Dev=>	1182	1417
1907	16	4954	7203 ^
	18	2982	6917 ^
	19	3436	7568 ^
	20	2246	5135 ^
	grp ave=>	3405	6706
	Std Dev=>	1143	1081

EXAMPLE 25 Hepatitis C- Sequences for RNAi Therapeutic DevelopmentExperiment 1Brief Introduction:

The hepatitis C virus (HCV) is the primary cause of non-A, non-B transfusion-associated hepatitis and accounts for more than 200 million 10 hepatitis cases worldwide. The HCV genome has a high degree of sequence variability. There are six major genotypes comprising more than

fifty subtypes and significant heterogeneity hallmarked by quasi-species has been found within patients. Great progress in understanding HCV replication has been made by using recombinant polymerases or cell-based subgenomic replicon systems. By using a replicon cell system,

5 HCV-specific siRNA has been demonstrated to be able to suppress HCV protein expression and RNA replication. Sequences of the 5' NTR and both structural and nonstructural genes have been targeted successfully. The highly conserved nature of the 3' NTR sequence makes it a highly attractive target for siRNA based therapy. However, no study has been

10 done to examine the feasibility of using the 3' NTR. Here we report the design and testing of several siRNAs that can inhibit HCV protein expression in the subgenomic replicon system. Exogenously synthesized HCV-specific siRNAs were transfected into the HCV replicon cell line as described below.

15

Cell culture and media:

The HCV replicon in hepatoma Huh7 cells was cultured in Dulbecco's Modified Eagle Media ("DMEM") (Invitrogen) containing 10% fetal calf serum (Invitrogen), 1% penicillin-streptomycin, 1% non-essential amino acids and 0.5 mg/mL Geneticin. Cells were grown to 75% confluence prior to splitting.

Western blot analysis:

Total cell lysates from replicon cells were harvested from replicon cells in 1× LDS Buffer (Invitrogen). The lysates were heated at 90 °C for 5 min in the presence of beta-mercaptoethanol before electrophoresis on a 10% Tris-Glycine polyacrylamide gel (Invitrogen). The protein was transferred to PVDF (Invitrogen) membrane. Following the transfer, the membrane was rinsed once with PBS containing 0.5% Tween-20 (PBS-Tween) and blocked in PBS-Tween containing 5% non-fat milk for 1 hr. After washing with PBS-Tween, the membrane was incubated with the

primary α -NS5A antibody (a gift from Dr. Chen Liu) at 1:1500 dilution for 1 hr at room temperature. Prior to incubation with HRP conjugated α -mouse IgG secondary antibody (Amersham) diluted 1:5000, the blot was washed in PBS-Tween 20. Following the secondary antibody incubation, the blot 5 was washed again and treated with ECL (Amersham) according to the manufacturer's protocol.

Northern blot:

Total cellular RNA was extracted by using the Rneasy® kit 10 (Qiagen). Northern blot analysis was done according to the protocol of Guo et al. Briefly, 5 μ g total RNA was electrophoresed through a 1.0% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane and immobilized by UV cross-linking (Stratagene). Hybridization was carried out using α -[³²P]CTP-labeled neomycin RNA in a 15 solution containing 50% deionized formamide, 5 \times SSC (750 mM sodium chloride, 750 mM sodium citrate), Denhardt's solution, 0.02 M sodium phosphate (pH 6.8), 0.2% sodium dodecyl sulfate ("SDS"), 100 μ g of sheared denatured salmon sperm DNA/ml, and 100 μ g of yeast RNA/ml, for 16 hr at 58 °C. The membranes were washed once in 2 \times SSC/0.1% 20 SDS for 30 min at room temperature and twice in 0.1 \times SSC/0.1% SDS for 30 min at 68 °C. Membranes were exposed to X-ray film.

Transfection of siRNA into replicon cells:

For transfection of siRNA into replicon cells the Lipofectamine® 25 2000 reagent (Invitrogen) was used according to the user manual. Briefly, 2 \times 10⁴ cells in 0.5 mL of DMEM was seeded in 24 well plates one day before the transfection. The indicated amount of siRNA was diluted in 50 μ L OptiMEM and mixed with diluted Lipofectamine® 2000 reagent (1 μ L in 30 50 μ L of Optimem). The mixture was incubated at room temperature for 20 min before being applied onto the cell monolayer. 48-72 hr after

transfection, cells were washed in PBS and lysed in 100 µL SDS sample buffer.

Table 14

<u>siRNA number</u>	<u>SEQ ID NO</u>	<u>HCV sequence</u>
#12	28	GCTAAACACTCCAGGCCAATACCTGTCTC
#22	29	TCCTTGTTGGCTCCATCTTACCTGTCTC
#32	30	GCTCCATCTTAGCCCTAGTCACCTGTCTC
#42	31	TCTTAGCCCTAGTCACGGCTACCTGTCTC
#52	32	CCTAGTCACGGCTAGCTGTGACCTGTCTC
#62	33	CTAGTCACGGCTAGCTGTGAACCTGTCTC
#72	34	CGTGAGCCGCTTGACTGCAGACCTGTCTC
#82	35	GCTGATACTGGCCTCTCTGCACCTGTCTC
#102	36	ACTGGCCTCTCTGCAGATCAACCTGTCTC

5

Several siRNAs comprising the HCV sequences identified above in Table 14 targeting the 3'UTR; siRNA #12 targeting the HCV NS5B gene (positive control); the identified HCV core siRNA (positive control); and the 10 identified lamin siRNA (negative control) were synthesized using the Silencer siRNA construction kit, Catalog # 1620 (Ambion Inc., Austin, Tex.). DNA oligonucleotides were synthesized by IDT (Coralville, Iowa).

Control siRNAs:

- 15 1. HCV core (positive control): SEQ ID NO:45
 2. #12, shown in Table 14, targeting the HCV NS5B gene, also a positive control
 3. lamin sequence (negative control): SEQ ID NO:46

20 Three siRNAs were used as controls: siRNA targeting the cellular gene Lamin for negative control; siRNA targeting the core sequence of HCV as a positive control; siRNA targeting the HCV NS5B gene as a positive control. Two concentrations of each siRNA (9 and 20 pmole) were used and the results were compared with transfection of no siRNA.

Accordingly, the Western Blots in FIG. 13 represent 0, 9, and 20 pmoles of the identified siRNAs. siRNA #22, 32, 42, 62, and 72 were notably active in repressing HCV NS5A protein expression. Presumably, HCV RNA level is also decreased based on the results obtained previously with positive 5 control siRNA for core. Several siRNAs had minimum effect at the concentrations tested and should be evaluated at higher concentrations. These include #12 (targeting NS5B), #102, #52, and #82.

Experiment 2

10 Experiment 2 was performed as described in Experiment 1 of Hepatitis C-Sequences for RNAi Therapeutic Development except that siRNAs R1-R8, comprising the sequences (and their complements) set forth in Table 15 below, were used in transfections. The Western Blot assay performed here was as described in Example 2, Experiment 1. The 15 control HCV core siRNA used as a positive control is the siRNA described in the previous HCV Experiment 1. All siRNAs were transfected at concentrations of 0, 9, and 20 pmole except the control "core" siRNA, which was transfected at levels of 0, 3, and 9 pmole. R1, R2, R3, R5, R7, and R8 all exhibited significant inhibition of HCV as can be seen in the 20 Western Blot, FIG. 14.

Table 15

siRNA	SEQ ID NO	HCV sequence
R1	37	CTGGCCTCTCTGCAGATCAAG
R2	38	TGCAGAGAGTGCTGATACTGG
R3	39	TGAGCCGCTTGACTGCAGAGA
R4	40	GAAAGGTCCGTGAGCCGCTT
R5	41	TAGCTGTGAAAGGTCCGTGAG
R6	42	TTAGCCCTAGTCACGGCTAGC
R7	43	TCCATCTTAGCCCTAGTCACG
R8	44	TTGGTGGCTCCATCTTAGCCC

All siRNAs evaluated map to the 3'UTR of the HCV genome and are conserved amongst HCV genotypes and quasi-species. SEQ ID NO:27 represents this 101 nt sequence of the HCV 3'UTR, sometimes referred to as the "X" region.

5

EXAMPLE 3

Silencing HBV replication and expression in a replication competent cell culture model

Brief description of cell culture model:

- 10 A human liver derived cell line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession
15 #s V01460 and J02203. Following internalization into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirrors HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core
20 particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg (Hepatitis B Surface Antigen) particles into the media of transfected cells. This transfection model reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression
25 and replication.

In this model, cells are co-transfected with the infectious molecular clone of HBV and the individual effector RNA constructs to be evaluated. The cells are then monitored for loss of HBV expression and replication as described below.

- 30 The following is an example of an experiment using eiRNA vectors encoding sequences derived from SEQ ID NO:1 and SEQ ID NO:5. The particular eiRNA vectors for this experiment are T7 RNA polymerase-

based (See, e.g., the teaching of WO 0063364, with respect to T7 dsRNA expression systems, as well as USSN 60/399,998P, filed 31-Jul-2002 and USSN 60/419,532, filed 18-Oct-2002) and encode hairpin RNA structures (especially desirable are, e.g., "forced" hairpin constructs, partial hairpins
5 capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002 and PCT/US2003/024028, filed 31-Jul-2003, as well as the "udderly" structured hairpins (e.g., multi-hairpin long dsRNA vectors and multi-short hairpin structures), hairpins with mismatched regions, and multiepitope constructs
10 as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, filed 20-Oct-2003). It is expected that similar results will be obtained using other expression and promoter systems, e.g., as described above, and/or vectors encoding alternative dsRNA structures (i.e. duplex).

15

Experimental Procedure: Transfection.

Huh7 cells are seeded into six-well plates such that they are between 80-90% confluence at the time of transfection. All transfections are performed using Lipofectamine™ (Invitrogen) according to the
20 manufacturer's directions. In this experiment, cells are transfected with 50 ng of the infectious HBV plasmid, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid below) 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:1 (described below) and 600 ng of an eiRNA vector encoding a
25 hairpin RNA comprised of sequences derived from SEQ ID NO:5 (described below). Control cells are transfected with 50 ng of the HBV plasmid and 1 µg of the T7 RNA polymerase expression plasmid. An inert filler DNA, pGL3-basic (Promega, Madison WI), is added to all transfections to bring total DNA/transfection up to 2.5 µg DNA.

30

Monitoring cells for loss of HBV expression.

Following transfection, cells are monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion and DNA-containing viral particle secretion. Cells are monitored by assaying

5 the media of transfected cells beginning at 2 days post dsRNA administration and every other day thereafter for a period of three weeks. The Auszyme ELISA, commercially available from Abbott Labs (Abbott Park, IL), is used to detect hepatitis B surface antigen (HBsAg). HBsAg is measured since HBsAg is associated not only with viral replication but also

10 with RNA polymerase II initiated transcription of the surface antigen cistron in the transfected infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only viral replication but also replication-independent synthesis of HBsAg. Secretion of virion particles containing encapsidated HBV

15 genomic DNA is also measured. Loss of virion particles containing encapsidated DNA is indicative of a loss of HBV replication. Analysis of virion secretion involves a technique that discriminates between naked, immature core particles and enveloped infectious HBV virions [6]. Briefly, pelleted viral particles from the media of cultured cells are subjected to

20 Proteinase K digestion to degrade the core proteins. Following inactivation of Proteinase K, the sample is incubated with RQ1 DNase (Promega, Madison, WI) to degrade the DNA liberated from core particles. The sample is digested again with Proteinase K in the presence of SDS to inactivate the DNase as well as to disrupt and degrade the infectious

25 enveloped virion particle. DNA is then purified by phenol/chloroform extraction and ethanol precipitated. HBV specific DNA is detected by gel electrophoresis followed by Southern Blot analysis.

Results will desirably indicate a 70-95% decrease in both HBsAg and viral particle secretion in the media of cells transfected with the HBV plasmid, T7 RNA polymerase expression plasmid and eiRNA constructs relative to cells transfected with only the HBV plasmid and T7 RNA polymerase expression plasmid.

Vectors used in experimentSequence of the T7 RNA polymerase gene

SEQ ID NO:47 represents the T7 RNA polymerase gene which is 5 cloned into a mammalian expression vector such as pCEP4 (Invitrogen, Carlsbad, CA). Cloning can be easily done by one skilled in the art. One skilled in the art would also be aware that a leader sequence with a Kozak sequence needs to be cloned in directly upstream from the T7 RNA polymerase gene.

10

eiRNA vector encoding RNA hairpin derived from SEQ ID NO:1

The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 3004-2950 (about 55 bp) of GenBank accession #s V01460 and J02203.

15 One region of the hairpin encodes the sense version of the sequences and the second region of the hairpin encodes the antisense version of this sequence. Hairpins can easily be designed and made by those skilled in the art.

20 eiRNA vector encoding RNA hairpin derived from SEQ ID NO:5

The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 730-786 of GenBank accession #s V01460 and J02203. The hairpin is designed as described for hairpin encoding sequences from SEQ ID NO:1.

25

EXPERIMENT 1Rationale for mouse models:

Chimpanzees represent the only animal model in which to study 30 human HBV infectivity. Mouse models are available, however, in which human HBV expression and replication occur. These models have been invaluable for the evaluation of anti-HBV therapeutic agents and have been shown to be a predictor for the efficacy of these agents in humans

- [4]. The first of these models are transgenic mouse models, in which the HBV genome or selected HBV genes are expressed [7,8]. Because HBV is integrated into the mouse genome, these animals serve as a model not only for viral replication but also for RT-independent expression of antigen.
- 5 A similar model exists in which replication competent HBV is expressed transiently from episomal HBV DNA. This model is created by introducing replication competent HBV DNA into mouse liver by hydrodynamic delivery [1]. Unlike the transgenic animals, these mice are not immunotolerant to HBV antigens and immune-mediated clearance of HBV transfected
- 10 hepatocytes can be studied.

Although woodchuck and duck models exist for the study of woodchuck hepatitis (WHBV) and duck hepatitis (DHBV) respectively, we have opted not to use these models for several reasons. 1) Human HBV cannot be studied in these models. As we are ultimately interested in

15 down-regulating expression of human HBV, use of these models would at some point necessitate the re-design and evaluation of vectors and/or RNAs specific for human HBV. 2) the mice are isogenic and therefore noise due to genetic variables within the system does not arise. 3) Unlike human HBV, there are no validated WHBV/DHBV cell culture models that

20 can be studied in parallel with their respective animal models.

The experiment described below utilizes hydrodynamic delivery as a method to co-deliver replication competent HBV^{yw} plasmid with the various effector dsRNA (eiRNA) expression vectors. Hydrodynamic delivery is ideal for this experiment because it results in efficient delivery of

25 nucleic acid to the liver [5]. Combination of the dsRNA effector plasmid and replication competent HBV plasmid into the same formulation increases the likelihood that both plasmids are taken up by the same cells. Because expressed effector dsRNA are present in the majority of cells bearing the replicating HBV plasmid, observed results can be attributed to

30 the performance of the effector plasmid rather than to differences in delivery efficiencies. This experiment demonstrates only that a particular eiRNA is efficacious in an infected liver. Formulation and delivery are not

addressed by this example. Formulation, dosing and delivery of the eiRNA vector are enabled in the example in which transgenic mice are used.

Experimental procedure:

- 5 Control B10.D2 mice are hydrodynamically injected with an infectious molecular clone of HBV (ayw subtype) consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1,2,3]. Following internalization into hepatocytes and nuclear localization, transcription of HBVayw plasmid
- 10 from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication [1]. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the sera of injected
- 15 animals. Animals are injected with four doses of the HBV replicon plasmid (1 µg, 3 µg, 5 µg, and 10 µg). These doses are chosen because they represent non-saturating doses capable of eliciting detectable expression of a reporter plasmid following hydrodynamic delivery. Animals are co-injected with the effector dsRNA expression vector (eiRNA) such that
- 20 animals in each group receive a 10-19 µg dose of a particular effector construct(s) such that the total DNA dose is 20 µg. For example in mice receiving the 3 µg dose of the HBV replicon, 17 µg of the chosen eiRNA vector(s) is injected for a total of 20 µg injected DNA. The amount of this dose is therefore dependent upon the dose of HBV plasmid used. Control
- 25 animals are injected with the HBV replicon but not with an eiRNA vector. Control mice are instead co-injected with an inert filler DNA, pGL3-basic (Promega, Madison, WI) such that the total amount of DNA in the formulation is 20 µg. eiRNA vectors in this study are the U6-based expression plasmids, e.g., Ambion, Inc., Austin, TX, USA. These vectors
- 30 encode short hairpin RNAs derived from SEQ ID NO:1 and SEQ ID NO:4. The exact sequences encoded by these vectors are described below. The vectors are co-injected in equal amounts (by weight). It is expected that

similar results will be obtained using other expression and promoter systems as described elsewhere herein and/or vectors encoding alternative structures (i.e. duplex).

Description of U6-based eiRNA vector encoding sequences derived
5 from SEQ ID NO:1: vector encodes a hairpin containing sequences mapping to coordinates 2905-2929 of accession #s V01460 and J02203 (i.e. the hairpin contains the sense and antisense version of this sequence, separated by a loop structure of TTCAAAAGA). Description of U6-based vector sequences can be found in Lee et al. [9]. The second eiRNA vector
10 used in this experiment encodes a hairpin derived from SEQ ID NO:4 and encodes sequences mapping to coordinates 1215-1239 of Accession # V01460 and J02203.

Liver samples are taken from injected animals on day 1 following injection and analyzed for the presence of HBV RNA. This time point has
15 been selected based on published results from Dr. Chisari's laboratory which detail the kinetics of HBVayw plasmid replication in mice following hydrodynamic delivery and demonstrates that peak RNA expression occurs in the liver on day 1 following hydrodynamic delivery [1]. The presence of HBV RNA in liver samples is ascertained by Northern blot
20 analysis. Liver tissue will be evaluated for the down-regulation of HBV RNA expression. In addition, serum will be collected from day 4 mice for measurement of HBVsAg and DNA-containing viral particles. Assays will be as described for the cell culture replicon experiment (Example 3) and as in Yang et al. [1]. Each vector and control group will be comprised of 2
25 sets of animals, each set corresponding to a collection time point. There are 5 animals in each set.

Results:

Mice that are injected with the HBV replicon and the eiRNA
30 constructs will have decreased HBV-specific RNA, and HBsAg and HBV viral particles as compared to the control animals. In individual animals, decreases will range from about 70% to near 100%.

EXPERIMENT 2

Transgenic mouse studies: Background.

We will be using the HBV transgenic mouse model developed in Dr. Chisari's laboratory [8]. These mice replicate appreciable amounts of HBV DNA and have demonstrated their utility as an antiviral screen that is a predictor of human efficacy [4]. These animals are also ideal in that they are a model for HBV-integrand-mediated expression of antigen and thus can serve as a model not only for viral replication but also for RT-independent expression of antigen. This is important as we are interested in targeting not only viral replication but integrant-mediated antigen expression as well.

These experiments differ from the hydrodynamic delivery experiments in that the effector plasmids are administered to animals using clinically relevant nucleic acid delivery methods. Effectiveness in this model demonstrates efficient delivery of the effector plasmids to mouse hepatocytes.

Experiment.

Mice described in reference [8] will be injected IV with a formulation containing the eiRNA vectors described in the hydrodynamic delivery example. These are the U6-based eiRNA vectors encoding hairpins containing sequences derived from SEQ ID NO:1 and SEQ ID NO:4.

25 Formulation of DNA to be injected.

DNA is formulated with trilactosyl spermine and cholesteryl spermine as described in PCT/US03/14288, "Methods for Delivery of Nucleic Acids", Satischchandran, filed 06-May-2003. Briefly, three formulations are made, all using a charge ratio of 1.2 (positive to negative charge). However, it should noted that formulations with charge ratios between 0.8 and 1.2 are all expected to exhibit efficacy. The DNA starting stock solution for each plasmid is 4 mg/ml. The two plasmid stock

solutions are mixed together in equal amounts such that each plasmid is at 2 mg/ml. This plasmid mixture is used for the final formulating. Formulation is as described in PCT/US03/14288 (above): Formulation A) 35% trilactosyl spermine, 65% cholesteryl spermine, Formulation B) 50% trilactosyl spermine, 50% cholesteryl spermine and Formulation C) 80% trilactosyl spermine, 20% cholesteryl spermine. All resultant formulations now contain each plasmid at 1 mg/ml.

Mice are IV injected with 100 µl formulated DNA. One group of mice receives Formulation A, a second group receives Formulation B and a third group receives Formulation C. Three groups of control mice are similarly injected with formulations containing a control DNA, pGL3Basic (Promega, Madison WI), Formulations D, E and F. Injections are carried out once a day for four consecutive days. Injecting for only 1-3 days is efficacious, however, more robust efficacy is seen with a four day injection protocol.

Following administration, HBV RNA and serum levels of HBsAg and DNA containing viral particles will be quantitated on days 5 and 9 post first injection. All analyses will be as described for the hydrodynamic delivery studies.

20

Results:

HBV-specific RNA levels, HBsAg and virus containing DNA particles will have decreased relative to controls in the Formulation A, B and C groups.

25

EXAMPLE #4

Silencing HBV replication and expression
in a replication competent cell culture model

Brief description of cell culture model:

30 A human liver derived cell line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the

viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession AF090840. Following internalization into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the media of transfected cells. This transfection model reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression and replication.

In this model, cells were co-transfected with the infectious molecular clone of HBV and an eiRNA construct. The cells were then monitored for loss of HBV expression and replication as described below.

The following is an example of an experiment that was performed using an eiRNA vector encoding sequences derived from both SEQ ID NO:1 and SEQ ID NO:2. The particular eiRNA vector used for this experiment is T7 RNA polymerase-based and encodes a duplex RNA of about 650 bp (See e.g., WO 00/63364, filed April 19, 2000). It is expected that similar results would be obtained using other expression and promoter systems as described elsewhere herein and/or vectors encoding alternative structures (i.e. duplex).

25 Experimental Procedure: Transfection.

Huh7 cells were seeded into six-well plates such that they were between 80-90% confluence at the time of transfection. All transfections were performed using LipofectamineTM (InVitrogen) according to the manufacturer's directions. In this experiment, cells were transfected with A) 50 ng of the infectious HBV plasmid adw subtype, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid in Example 3), and 1.5 µg of the HBV-specific eiRNA vector (described below); B) 50 ng of the

infectious HBV plasmid, 1 µg of the T7 RNA polymerase expression plasmid and 1.5 µg of an irrelevant dsRNA expression vector; C) 125 ng of the infectious HBV plasmid, 1 µg of the T7 RNA polymerase expression plasmid and 1.4 µg of the HBV-specific eiRNA vector; and D) 125 ng of the 5 infectious HBV plasmid, 1 µg of the T7 RNA polymerase expression plasmid and 1.4 µg of an irrelevant dsRNA expression vector. All transfections were carried out in duplicate. In this experiment transfections B and D served as controls. Four days post-transfection, media was removed from transfected cells and assayed for the presence of HBsAg 10 (see below). Media from untransfected cells was also assayed as a background control.

Monitoring cells for loss of HBV expression.

Following transfection, cells were monitored for the loss or 15 reduction in HBV expression and replication by measuring HBsAg secretion. Cells were monitored by assaying the media of transfected cells (and a media control) at 4 days post-dsRNA administration. The Auszyme ELISA, commercially available from Abbott Labs (Abbott Park, IL), was used to detect hepatitis B surface antigen (HBsAg). HBsAg was measured 20 since it is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only viral replication but also replication-independent synthesis of HB sAg.

25

Results:

Cells transfected with the HBV-specific eiRNA construct exhibited an 82-93% decrease in HBsAg at the four-day timepoint relative to the control transfections.

30

HBV-specific eiRNA used in this experiment

The eiRNA vector encodes a dsRNA mapping to coordinates 2027-2674 of GenBank Accession # AF090840. The sequence therefore includes sequences derived from both SEQ ID NO:1 and SEQ ID NO:2.

- 5 More specifically, the sequence includes all of SEQ ID NO:2 and 134 bp derived from SEQ ID NO:1.

EXAMPLE #5The down-regulation of HCV in a cell culture replicon model10 Brief description

In this experiment, a cell line is created which expresses functional HCV replicons. Creation of the cell line is as detailed in Lohmann et al. [10]. In this experiment Huh7 cells are used as the parental cell line but in theory any human hepatocyte derived cell line can be used. The cells are 15 then transfected with an HCV specific eiRNA vector. The presence of HCV-specific RNA is ascertained by Northern blot analysis as described in Lohmann et al. [10] at days 3-7 post-transfection of eiRNA.

Experimental protocol: Transfection.

20 Huh7 cells expressing HCV replicons are seeded into six-well plates such that they are between 80-90% confluence at the time of transfection. All transfections are performed using LipofectamineTM (InVitrogen) according to the manufacturer's directions. In this experiment, cells are transfected with 1 µg of a T7 RNA polymerase expression 25 plasmid (plasmid described in Example 3) and 1.5 µg of a T7-based eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:11 (vector described at end of example). Control cells are transfected with 1 µg T7 RNA polymerase expression plasmid and 1.5 µg of the HBV-specific (SEQ ID NO:1 specific) T7-based eiRNA vector 30 described in Example 3. Untransfected replicon-expressing HuH 7 cells are included as a second control. Each transfection mix is made such that ten transfections can be performed/mix resulting in a total of 20

transfections (10 per mix). At days 3, 4, 5, 6, and 7, two wells of cells/each transfection are lysed and RNA is extracted using standard techniques. Samples are analyzed simultaneously by Northern blot analysis for the presence of HCV-specific RNA as described in Lohmann et al. [10].

5

Results

Cells transfected with the HCV-specific eiRNA vector will show decreased HCV-specific RNA levels relative to the control cells at every time-point analyzed.

10

HCV-specific eiRNA vector.

The eiRNA vector is T7-based and encodes a hairpin RNA. One side of the hairpin comprises SEQ ID NO:48.

This sequence is followed by a loop structure of 9 Ts. The second 15 side of the hairpin contains a sequence that is complementary to the first side of the hairpin. One skilled in the art can easily design and construct hairpin constructs. Note: it is anticipated that other types of eiRNA vectors driven by other promoters and encoding other types of RNA structures will have similar effects.

20

EXAMPLE #6

Treatment of an HBV/HCV co-infection

Brief description

In this example, cells that are replicating both HBV and HCV 25 replicons are transfected with an eiRNA vector that encodes both HBV and HCV-specific eiRNA.

Experimental protocol:

Creation of cell lines that contain both HBV and HCV replicons.

30 HuH 7 cells are first engineered to express functional HCV replicons. Creation of the cell line is as detailed in Lohmann et al. [10]. After cell line establishment, the cells are transfected with an infectious

HBV replicon plasmid as described in Example 3 and below in the "Transfection of cells" section. In this example, the replicon is derived from the virus sequence found in Gen Bank Accession #s V01460 and J02203. Theoretically, it is also possible to first create a cell line that stably

5 expresses the HBV replicon and then use this cell line to create one that also expresses HCV replicons. It is also possible to transfect the cells simultaneously with both the HBV and HCV replicons and select and expand cells that are replicating both HBV and HCV replicons.

10 Transfection of cells.

In this example, the HBV and HCV eiRNAs are encoded by separate cistrons within the same vector. However, similar results are expected if the eiRNAs are encoded within the same cistron or provided by separate vectors. In this example, transcription from each cistron is driven

15 by the T7 RNA polymerase promoter and T7 RNA polymerase. Each promoter is followed by a hairpin eiRNA which in turn is followed by a T7 terminator (FIG. 1). The cistrons in this example are converging but one could also use diverging cistrons. It should also be noted that one could use other expression systems (including viral) to produce these RNAs and

20 one could also use other promoters, e.g., as described elsewhere herein, to drive expression of these RNAs without significantly affecting efficacy. Selection of the appropriate expression systems and promoters is within the skill in this art. Also one could express other eiRNA structures, e.g., as described elsewhere herein, as well as others, described in the literature in

25 this area. In this example, the HBV eiRNA vector encodes sequences derived from SEQ ID NO:1 and the HCV eiRNA vector encodes sequences derived from SEQ ID NO:11. Description of vector inserts is located at the end of this example.

Huh7 cells are seeded into six-well plates such that they are

30 between 80-90% confluence at the time of transfection. All transfections are performed using LipofectamineTM (Invitrogen) according to the manufacturer's directions. In this experiment, cells are transfected with 50

ng of the infectious HBV plasmid, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid is in Example 3), 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:1 (described below and in Example 3), and 600 ng of an
5 eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:11 (described below). Control cells are transfected with 50 ng of the HBV plasmid and 1 µg of the T7 RNA polymerase expression plasmid. An inert filler DNA, pGL3-basic (Promega, Madison WI), is added to all transfections where needed to bring total DNA/transfection up to 2.5
10 µg DNA. Each transfection mix is made such that ten transfections can be performed/mix resulting in a total of 20 transfections (10 per mix).

Analyses.

Following transfection, cells are monitored for the loss or reduction
15 in HBV expression and replication by measuring HBsAg secretion and DNA-containing viral particle secretion. Cells are monitored by assaying the media of transfected cells beginning at 2 days post dsRNA administration and every other day thereafter for a period of three weeks. The Auszyme ELISA, commercially available from Abbott Labs (Abbott Park, IL), is used to detect hepatitis B surface antigen (HBsAg). HBsAg is measured since it is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only
20 viral replication but also replication-independent synthesis of HBsAg. Secretion of virion particles containing encapsidated HBV genomic DNA is also measured. Loss of virion particles containing encapsidated DNA is indicative of a loss of HBV replication. Analysis of virion secretion involves a technique that discriminates between naked, immature core particles
25 and enveloped infectious HBV virions [6]. Briefly, pelleted viral particles from the media of cultured cells are subjected to Proteinase K digestion to degrade the core proteins. Following inactivation of Proteinase K, the
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sample is incubated with RQ1 DNase (Promega, Madison, WI) to degrade the DNA liberated from core particles. The sample is digested again with Proteinase K in the presence of SDS to inactivate the DNase as well as to disrupt and degrade the infectious enveloped virion particle. DNA is then 5 purified by phenol/chloroform extraction and precipitated. HBV specific DNA is detected by gel electrophoresis followed by Southern Blot analysis.

At days 3, 4, 5, 6 and 7, two wells of cells/each transfection (experimental and control) are lysed and RNA is extracted using standard techniques. Samples are also analyzed by Northern blot analysis for the 10 presence of HCV-specific RNA as described in Lohmann et al. [10].

Results.

Cells transfected with the HBV-HCV-specific eiRNA vector will show decreased HCV-specific RNA levels relative to the control cells at every 15 time-point analyzed. In addition, the levels of HBsAg and HBV viral particles will also decrease relative to the control transfections.

HCV-specific eiRNA sequence.

The eiRNA vector is T7-based and encodes a hairpin RNA. One 20 side of the hairpin comprises SEQ ID NO:48.

This sequence is followed by a loop structure of 9 Ts. The second side of the hairpin contains a sequence that is complementary to the first side of the hairpin. One skilled in the art can easily design and construct hairpin constructs. Note: it is anticipated that other types of eiRNA vectors 25 driven by other promoters and encoding other types of RNA structures, including various hairpin structures will have similar effects. Especially desirable are, e.g., "forced" hairpin constructs, partial hairpins capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002 and 30 PCT/US2003/024028, filed 31-Jul-2003, as well as the "udderly" structured hairpins (e.g., multi-hairpin long dsRNA vectors and multi-short hairpin structures), hairpins with mismatched regions, and multiepitope constructs

as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, filed 20-Oct-2003, as well as a variety of other dsRNA structures known to those of skill in the art.

5 HBV-specific eiRNA-SEQ ID NO:1

The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 3004-2950 (About 55 bp) of GenBank accession #s V01460 and J02203. One region of the hairpin encodes the sense version of the sequences and 10 the second region of the hairpin encodes the antisense version of this sequence. Hairpins can easily be designed and made by those skilled in the art.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

PACHUK et al.

CASE NO: 26788-024

SERIAL NO: 10/560,377

GROUP ART UNIT: Unknown

FILED: DECEMBER 12, 2005

EXAMINER: Unknown

FOR: CONSERVED HBV AND HCV
SEQUENCES USEFUL FOR GENE
SILENCING

PRELIMINARY AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicant respectfully requests that the Examiner enter the amendments below prior to examining the above-identified application on its merits.

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims that begin on page 59 of this paper.

Amendments to the Drawings begin on page 72 of this paper and include attached replacement sheets.

Remarks begin on page 73 of this paper.

Amendments to the Specification:

Replace the paragraph beginning at page 1, line 5, with the following amended paragraph:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a 371 of PCT Application No. PCT/US2004/019229, filed June 10, 2004, now pending, which claims priority from U.S. Provisional Application No. 60/478,076, filed June 12, 2003, each of which is incorporated by reference herein in their entireties. This application also claims priority from U.S. Provisional Application No. 60/638,294, filed December 22, 2004, which is incorporated by reference herein in its entirety.

Replace the section beginning at page 3, line 29, and ending at page 6, line 26, with the following amended section:

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6, or more double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T. In a preferred method, three or four dsRNA effector molecules, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T; are administered to an *in vivo* mammalian cell. The double-stranded RNA effector molecules may be prepared exogenously and administered into a mammalian cell or expressed intracellularly in a mammalian cell from a double-stranded RNA expression vector, i.e., an expression vector engineered to express a dsRNA effector molecule in a mammalian cell. In a preferred method, at least three or four dsRNA effector molecules, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and

SEQ ID NO:49; wherein U is substituted for T; are encoded in a dsRNA expression vector which is administered to an *in vivo* mammalian cell.

A composition for inhibiting the expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising at least one, preferably 2, 3, 4, 5, 6 or more double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T. In a preferred composition, at least three or four dsRNA effector molecules are included, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T. The double-stranded RNA effector molecules may be prepared exogenously and the composition comprising two, three, four, five, six, or more dsRNA effector molecules administered into a mammalian cell, or the composition may comprise one or more dsRNA expression constructs capable of expressing in a mammalian cell two, three, four, five, six or more of said dsRNA effector molecules. In a preferred composition, three or four dsRNA effector molecules, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T, are encoded in a dsRNA expression vector.

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell at least two, preferably 3, 4, 5, 6 or more, double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In a preferred method, said dsRNA effector molecules will comprise 3 or 4 sequences selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:59, and SEQ ID NO:62; wherein U is substituted for T. The double-stranded RNA effector molecules may be stem-loop or hairpin structures and/or duplex double-stranded RNA molecules. The double-stranded RNA effector molecules may be prepared exogenously and the two, three, four, five, six, or more dsRNA effector molecules administered into a mammalian cell, or one or more

dsRNA expression constructs capable of expressing in a mammalian cell two, three, four, five, six or more of said dsRNA effector molecules may be administered.

A composition for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising at least two double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In a preferred composition, three or four of said dsRNA effector molecules will be included, or encoded in an expression vector, comprising sequences selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:59, and SEQ ID NO:62; wherein U is substituted for T. The double-stranded RNA effector molecules may be prepared exogenously and the composition will comprise two, three, four, five, six, or more of said dsRNA effector molecules for administration into a *in vivo* mammalian cell, or the composition may comprise one or more dsRNA expression constructs capable of expressing in a mammalian cell two, three, four, five, six or more of said dsRNA effector molecules.

In another aspect the invention relates to methods and compositions for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell at least two, preferably 3, 4, 5, 6 or more, double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 50; SEQ ID NO: 51; SEQ ID NO: 52; SEQ ID NO:53; SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56; SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO: 60; SEQ ID NO:61; and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T.

A polynucleotide sequence comprising SEQ ID NO:49.

A method for inhibiting expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering to said cell at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6, or more double-stranded RNA effector molecules, comprising (a) an RNA sequence equivalent to a

hepatitis C virus DNA coding strand sequence selected from the group consisting of sequence position 9510-9531, 9510-9533, 9510-9534, 9510-9535, 9510-9536, 9514-9534, 9514-9535, 9514-9536, 9514-9539, 9514-9540, 9514-9542, 9517-9539, 9517-9540, 9517-9542, 9517-9544, 9518-9539, 9518-9540, 9518-9542, 9518-9544, 9520-9540, 9520-9542, 9520-9544, 9520-9548, 9521-9542, 9521-9544, 9521-9548, 9521-9549, 9522-9542, 9522-9544, 9522-9548, 9522-9549, 9527-9548, 9527-9549, 9527-9551, 9527-9552, 9527-9553, 9527-9555, 9528-9548, 9528-9549, 9528-9551, 9528-9552, 9528-9553, 9528-9555, 9530-9551, 9530-9552, 9530-9553, 9530-9555, 9530-9557, 9530-9558, 9532-9552, 9532-9553, 9532-9555, 9532-9557, 9532-9558, 9532-9559, 9532-9560, 9537-9557, 9537-9558, 9537-9559, 9537-9560, 9537-9561, 9537-9564, 9538-9558, 9538-9559, 9538-9560, 9538-9561, 9538-9564, 9538-9566, 9541-9561, 9541-9564, 9541-9566, 9541-9568, 9541-9569, 9543-9564, 9543-9566, 9543-9568, 9543-9569, 9543-9571, 9545-9566, 9545-9568, 9545-9569, 9545-9571, 9545-9573, 9546-9564, 9546-9566, 9546-9569, 9546-9571, 9546-9573, 9547-9568, 9547-9569, 9547-9571, 9547-9573, 9547-9575, 9550-9571, 9550-9573, 9550-9575, 9550-9577, 9550-9578, 9554-9575, 9554-9577, 9554-9578, 9554-9580, 9556-9577, 9556-9578, 9556-9580, 9556-9584, 9562-9584, 9562-9586, 9562-9587, 9562-9588, 9562-9589, 9563-9584, 9563-9586, 9563-9587, 9563-9588, 9563-9589, 9563-9591, 9565-9586, 9565-9587, 9565-9588, 9565-9589, 9565-9591, 9565-9593, 9567-9587, 9567-9588, 9567-9589, 9567-9591, 9567-9593, 9567-9595, 9570-9591, 9570-9593, 9570-9595, 9570-9596, 9570-9598, 9572-9593, 9572-9595, 9572-9596, 9572-9598, 9574-9595, 9574-9596, 9574-9598, 9574-9601, 9576-9596, 9576-9598, 9576-9601, 9576-9604, 9579-9601, 9579-9604, 9581-9601, 9581-9604, and 9583-9604 and (b) an RNA sequence which is the reverse complement of the selected sequence equivalent to the hepatitis C virus DNA coding strand sequence. In some embodiments, said RNA sequences (a) and (b) are linked by a loop sequence and the double-stranded RNA effector molecule(s) forms a stem-loop or hairpin dsRNA structure. In some aspects, said double-stranded RNA effector molecule(s) are duplex dsRNAs, formed from two separate RNA strands. In some aspects, the method involves administering to a mammalian cell an expression construct encoding one, two, three, four, five or more of said dsRNA effector molecules. In some embodiments designed to target the HCV minus strand, the dsRNA effector molecule will comprise (a) an RNA sequence corresponding to a hepatitis C virus

DNA coding strand sequence as specified above, and (b) the reverse complement of said RNA sequence, optionally linked by a loop sequence. In some embodiments, the dsRNA effector molecule(s) is encoded by an expression construct.

In some aspects the invention relates to a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6 or more double-stranded RNA effector molecules, or a dsRNA expression construct capable of transcribing one, 2, 3, 4, 5, 6 or more of said dsRNA effector molecules in an *in vivo* mammalian cell, each of said dsRNA effector molecules comprising (a) an RNA sequence equivalent to a hepatitis C virus DNA coding strand sequence selected from the group consisting of sequence position 9510-9531, 9510-9533, 9510-9534, 9510-9535, 9510-9536, 9514-9534, 9514-9535, 9514-9536, 9514-9539, 9514-9540, 9514-9542, 9517-9539, 9517-9540, 9517-9542, 9517-9544, 9518-9539, 9518-9540, 9518-9542, 9518-9544, 9520-9540, 9520-9542, 9520-9544, 9520-9548, 9521-9542, 9521-9544, 9521-9548, 9521-9549, 9522-9542, 9522-9544, 9522-9548, 9522-9549, 9527-9548, 9527-9549, 9527-9551, 9527-9552, 9527-9553, 9527-9555, 9528-9548, 9528-9549, 9528-9551, 9528-9552, 9528-9553, 9528-9555, 9530-9551, 9530-9552, 9530-9553, 9530-9555, 9530-9557, 9530-9558, 9532-9552, 9532-9553, 9532-9555, 9532-9557, 9532-9558, 9532-9559, 9532-9560, 9537-9557, 9537-9558, 9537-9559, 9537-9560, 9537-9561, 9537-9564, 9538-9558, 9538-9559, 9538-9560, 9538-9561, 9538-9564, 9538-9566, 9541-9561, 9541-9564, 9541-9566, 9541-9568, 9541-9569, 9543-9564, 9543-9566, 9543-9568, 9543-9569, 9543-9571, 9545-9566, 9545-9568, 9545-9569, 9545-9571, 9545-9573, 9546-9564, 9546-9566, 9546-9569, 9546-9571, 9546-9573, 9547-9568, 9547-9569, 9547-9571, 9547-9573, 9547-9575, 9550-9571, 9550-9573, 9550-9575, 9550-9577, 9550-9578, 9554-9575, 9554-9577, 9554-9578, 9554-9580, 9556-9577, 9556-9578, 9556-9580, 9556-9584, 9562-9584, 9562-9586, 9562-9587, 9562-9588, 9562-9589, 9563-9584, 9563-9586, 9563-9587, 9563-9588, 9563-9589, 9563-9591, 9565-9586, 9565-9587, 9565-9588, 9565-9589, 9565-9591, 9565-9593, 9567-9587, 9567-9588, 9567-9589, 9567-9591, 9567-9593, 9567-9595, 9570-9591, 9570-9593, 9570-9595, 9570-9596, 9570-9598, 9572-9593, 9572-9595, 9572-9596, 9572-9598, 9574-9595, 9574-9596, 9574-9598, 9574-9601, 9576-9596, 9576-9598, 9576-9601, 9576-9604, 9579-9601, 9579-9604, 9581-9601, 9581-9604, and 9583-9604 and (b) the reverse complement of said selected

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RNA sequence equivalent to the hepatitis C virus DNA coding strand sequence. In some embodiments, said RNA sequences (a) and (b) are linked by a loop sequence, and the double-stranded RNA effector molecule(s) is a single RNA strand which forms a stem-loop or hairpin dsRNA structure. In other embodiments, the dsRNA effector molecule(s) is a duplex dsRNA molecule formed from two separate strands of RNA.

In another aspect, the invention relates to compositions for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6 or more double-stranded RNA effector molecules, or a dsRNA expression construct capable of expressing one, 2, 3, 4, 5, 6 or more of said dsRNA effector molecules in an *in vivo* mammalian cell, each of said dsRNA effector molecules comprising (a) a sequence selected from the group consisting of SEQ ID NO: 63; SEQ ID NO: 64; SEQ ID NO: 65; SEQ ID NO: 66; SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69; SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73; SEQ ID NO: 74; SEQ ID NO: 75; and SEQ ID NO: 76; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In certain preferred embodiments, the sequence is selected from the group consisting of SEQ ID NO: 72; SEQ ID NO: 73; SEQ ID NO: 74; SEQ ID NO: 75; and SEQ ID NO: 76.

In another aspect, the invention relates to methods for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6 or more double-stranded RNA effector molecules, or a dsRNA expression construct capable of expressing one, 2, 3, 4, 5, 6 or more of said dsRNA effector molecules in an *in vivo* mammalian cell, each of said dsRNA effector molecules comprising (a) a sequence selected from the group consisting of SEQ ID NO: 63; SEQ ID NO: 64; SEQ ID NO: 65; SEQ ID NO: 66; SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69; SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73; SEQ ID NO: 74; SEQ ID NO: 75; and SEQ ID NO: 76; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In certain preferred

embodiments, the sequence is selected from the group consisting of SEQ ID NO:72; SEQ ID NO: 73; SEQ ID NO:74; SEQ ID NO: 75; and SEQ ID NO:76.

In another aspect, the invention relates to a polynucleotide sequence comprising an RNA sequence equivalent to and/or complementary to a hepatitis C virus DNA coding strand sequence selected from the group consisting of sequence position 9510-9531, 9510-9533, 9510-9534, 9510-9535, 9510-9536, 9514-9534, 9514-9535, 9514-9536, 9514-9539, 9514-9540, 9514-9542, 9517-9539, 9517-9540, 9517-9542, 9517-9544, 9518-9539, 9518-9540, 9518-9542, 9518-9544, 9520-9540, 9520-9542, 9520-9544, 9520-9548, 9521-9542, 9521-9544, 9521-9548, 9521-9549, 9522-9542, 9522-9544, 9522-9548, 9522-9549, 9527-9548, 9527-9549, 9527-9551, 9527-9552, 9527-9553, 9527-9555, 9528-9548, 9528-9549, 9528-9551, 9528-9552, 9528-9553, 9528-9555, 9530-9551, 9530-9552, 9530-9553, 9530-9555, 9530-9557, 9530-9558, 9532-9552, 9532-9553, 9532-9555, 9532-9557, 9532-9558, 9532-9559, 9532-9560, 9537-9557, 9537-9558, 9537-9559, 9537-9560, 9537-9561, 9537-9564, 9538-9558, 9538-9559, 9538-9560, 9538-9561, 9538-9564, 9538-9566, 9541-9561, 9541-9564, 9541-9566, 9541-9568, 9541-9569, 9543-9564, 9543-9566, 9543-9568, 9543-9569, 9543-9571, 9545-9566, 9545-9568, 9545-9569, 9545-9571, 9545-9573, 9546-9564, 9546-9566, 9546-9569, 9546-9571, 9546-9573, 9547-9568, 9547-9569, 9547-9571, 9547-9573, 9547-9575, 9550-9571, 9550-9573, 9550-9575, 9550-9577, 9550-9578, 9554-9575, 9554-9577, 9554-9578, 9554-9580, 9556-9577, 9556-9578, 9556-9580, 9556-9584, 9562-9584, 9562-9586, 9562-9587, 9562-9588, 9562-9589, 9563-9584, 9563-9586, 9563-9587, 9563-9588, 9563-9589, 9563-9591, 9565-9586, 9565-9587, 9565-9588, 9565-9589, 9565-9591, 9565-9593, 9567-9587, 9567-9588, 9567-9589, 9567-9591, 9567-9593, 9567-9595, 9570-9591, 9570-9593, 9570-9595, 9570-9596, 9570-9598, 9572-9593, 9572-9595, 9572-9596, 9572-9598, 9574-9595, 9574-9596, 9574-9598, 9574-9601, 9576-9596, 9576-9598, 9576-9601, 9576-9604, 9579-9601, 9579-9604, 9581-9601, 9581-9604, and 9583-9604.

Applicants' invention further provides a method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and

SEQ ID NO:10; wherein U is substituted for T. In a preferred embodiment of the method, effector sequences from more than one SEQ ID sequence may be administered to the same cell, and/or more than one effector sequence from within the same SEQ ID sequence may be administered to the same cell.

Applicants further provide a method for inhibiting expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering to said cell a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:27; wherein U is substituted for T. In a preferred embodiment of this aspect of the method, effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 may be administered to the same cell; or from both SEQ ID NO: 11 and SEQ ID NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from each of SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO:27, are administered to the same cell; and/or more than one effector molecule from within the same SEQ ID NO may be administered to the same cell.

Applicants further provide a method for inhibiting expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in the same *in vivo* mammalian cell, comprising administering to said cell a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:27; wherein U is substituted for T. In preferred embodiments of this aspect of the invention, effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 may be administered to the same cell; and/or effector molecules from both SEQ ID NO:11 and SEQ ID NO:12; or from both SEQ ID NO: 11 and SEQ ID NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from SEQ ID NO: 11, SEQ ID NO:12 and SEQ ID NO:27; may be

administered to the same cell; and/or more than one effector molecules from within the same SEQ ID NO may be administered to the same cell.

Applicants further provide a composition for inhibiting the expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO is present in the composition.

Applicants further provide a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12 and SEQ ID NO:27; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 are present in the composition; or from both SEQ ID NO: 11 and SEQ ID NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from each of SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO:27, are present in the same composition, and/or wherein more than one effector molecule from within the same SEQ ID NO may be present in the composition.

Applicants further provide a composition for inhibiting the expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in a single *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12 and SEQ ID

NO:27; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12; or from both SEQ ID NO: 11 and SEQ ID NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from each of SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO:27, are present in the composition; and/or wherein more than one effector sequence from within the same SEQ ID NO may be present in the composition.

In particularly preferred embodiments of the above methods and compositions of the invention, the polynucleotide sequence is present within a double-stranded region of an RNA, and the mammalian cell is a human cell.

Further provided are compositions for inhibiting the expression of a polynucleotide sequence of hepatitis B virus and/or a polynucleotide sequence of hepatitis C virus in mammalian cells, wherein said compositions comprise an at least 19 contiguous nucleotide sequence selected from within SEQ ID NO:1 through SEQ ID NO:12, and SEQ ID NO:27; the complement sequences of said SEQ ID NO:1 through SEQ ID NO:12, and SEQ ID NO: 27 sequences, and mixtures of these sequences. In this embodiment of the invention, the "an at least 19 contiguous nucleotide sequence" is preferably DNA, and the mammalian cell is preferably human. Also provided are expression constructs comprising any of the aforementioned compositions and a mammalian cell comprising said expression constructs.

Replace the paragraph beginning at page 6, line 27, with the following amended paragraph:

Another aspect provides for a polynucleotide sequence comprising a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for polynucleotide sequence comprising nucleotides 1-19, 1-20, 1-21, 2-20, 2-21, or 3-21 of a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for a polynucleotide sequence comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from SEQ ID NO:27 through SEQ ID NO:44.

Replace the paragraph beginning at page 7, line 4, with the following amended paragraph:

Another aspect provides a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in a mammalian cell, comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:27; wherein U is substituted for T.

Add, at page 7, line 9, the following new paragraph:

In various aspects of the foregoing methods and compositions, the *in vivo* mammalian cell is an *in vivo* human cell.

Replace the paragraph beginning at page 8, line 6, with the following amended paragraph:

SEQ ID NO:48 represents a 5' segment of the hepatitis C virus sequence (corresponds to positions 36 to 358 in Genbank Accession Number AJ238799, with 2 base changes, C to G at AJ238799 position 204 and G to A at AJ238799 position 357).

Add, at page 8, line 8, the following new section:

SEQ ID NO:49 represents an eiRNA (shRNA) molecule to a conserved HBV sequence.

SEQ ID NO:50 through SEQ ID NO:62 represent the first 21 nucleotides of SEQ ID NOs: 14-23, 25-26, and 49.

SEQ ID NO:63 through SEQ ID NO:71 represent the first 21 nucleotides of SEQ ID NOs: 28-36.

SEQ ID NO:72 through SEQ ID NO:76 represent highly conserved coding region sequitopes from the 5' and 3' untranslated regions of HCV.

SEQ ID NO:77 through SEQ ID NO:109 represent highly conserved HCV sequences from the 5' UTR of the HCV (SEQ ID NO: 11).

Add, at page 9, line 23, the following new paragraph:

Figure 15 is a table of additional conserved HCV genome sequence segments suitable for generating dsRNA effector molecules which inhibit the expression of polynucleotide sequences of hepatitis C virus, including expressed shRNA for gene silencing. Each sequence represents a DNA coding strand sequence in standard 5' to 3' polarity which (together with its reverse complement) can be utilized to transcribe or design a double-stranded RNA effector molecule, e.g., an shRNA or duplex dsRNA molecule targeted to degrade the negative strand of HCV RNA. E.g., an DNA sequence, followed by a loop sequence (e.g., a 9 base loop sequence as described elsewhere herein), followed by the reverse complement of the sequence given in the table, may be incorporated into an expression construct under the control of an appropriate promoter. The shRNA molecule transcribed from such an expression construct is expected to inhibit expression of HCV polynucleotide sequences and/or mediate dsRNA silencing of HCV. For example, in the case of the 22 base sequence shown for positions 9545-9566, a construct is made to contain a 53 bp insert, comprising the 22 base sequence of 9545-9566, a linker or loop sequence, and the reverse complement of the 9545-9566 sequence, preferably under the control of an RNA polymerase III promoter and ending with an RNA polymerase III terminator, e.g., a run of 4, 5, or more T residues. The RNA equivalent of this sequence, having U's instead of T's, would read (in the 5' to 3' direction):

AAAGGUCCGUGAGCCGCUUGAC-XXXXXX-XX-GUCAAGCGGUCACGGACCUU
U

where X represents bases of the loop that are unable to form stable base pairs with any other portion of the 53 bp shRNA sequence. The loop may vary considerably, however, as to both length and nucleotide sequence, so long as the formation of the double-stranded "stem" region of the hairpin is not adversely affected. Thus, in

expression constructs that are the subject of this invention, the sequence element above beginning at the end which reads 5' AAAGGT is cloned into an appropriate vector downstream from and operably linked to the promoter. As described elsewhere herein, in preferred embodiments, two, three, four, five, six, seven, or more of the shRNAs encoded by these sequences, optionally, together with other anti-HCV, and/or HBV sequences described herein, are coded into and expressed by a single dsRNA expression vector. In one aspect, each of said multiple stem-loop or shRNA molecules is encoded in a single expression vector within a different expression cassette, each operably linked to a promoter and a terminator, preferably a polymerase III promoter, which may be the same or different. In another aspect, two or more hairpin dsRNA molecules may be expressed from a single promoter, as e.g., a bi-fingered molecule in which a single transcribed RNA strand comprises two such shRNA sequences separated by an unrelated linker sequence. Such constructs, in which a single expression vector provides a mammalian cell with two, three, four, five or more independent dsRNA effector molecules against an HCV and/or HBV target polynucleotide, are particularly desirable for pharmaceutical applications. An alternative means of dsRNA-mediated silencing may be accomplished by preparing shRNAs or duplex dsRNAs corresponding to the identified sequences by chemical synthesis or *in vitro* expression and delivering them into a cell in order to achieve inhibition of HCV and/or HBV polynucleotide sequences.

Replace the paragraph beginning at page 10, line 30, with the following amended paragraph:

In GenBank version 132.0 there are more than 4500 HBV sequences and 340 HBV complete genome sequences (317 Human isolates, 22 isolates from other primates and one woodchuck HBV isolate). This variability constitutes a serious challenge for sequence-specific pharmaceutical approaches such as RNAi. In order to identify conserved sequences suitable for RNAi applications, a comparison between all the complete genomes was carried out using a modified version of ClustalW. Two multiple alignment schemes were generated: the first included all 339 HBV complete genome sequences and the second was limited to all Human

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HBV isolates. The multiple alignment results were parsed and a table that included scores for sequence conservation at each position in the HBV genome was generated. A sliding window search to identify the longest region of sequence conservation larger than 19 nt in length was created. Three major conserved regions were identified and mapped to GenBank accession no.: AF090840, a Human HBV isolate. The conserved HBV sequences were screened against Genbank sequences of both human genomic and cDNA libraries (Human chromosomes database). It was found that 21 nucleotide and longer segments selected as a permuted "window" from within the conserved regions were unique to HBV, i.e. no perfect sequence matches exist between any 21 nt or longer HBV conserved segments and the available sequence databases of human chromosomal and RNA sequences. For human therapeutic purposes, assuring that homologous human sequences are not inadvertently silenced is as important as selecting conserved viral sequences for RNAi.

Replace the paragraph beginning at page 12, line 13, with the following amended paragraph:

In GenBank version 134.0 there are more than 20,000 HCV sequences and 93 HCV complete genome sequences. A comparison between all the complete genomes was carried out using a modified version of ClustalW. The multiple alignment result was parsed and a table that included scores for sequence conservation at each position in the HCV genome was generated. A sliding window search to identify the longest region of sequence conservation larger than 19 nt in length was created. Three major conserved regions were identified and mapped to GenBank RefSeq (reference sequence) accession no.: NC_004102 this is GenBank annotated HCV complete genome. The three major conserved regions include a portion of the 3' untranslated region of the virus, already described in the literature to be well-conserved among viral isolates. See, e.g., US Patent 5,874,565, "Nucleic Acids Comprising a Highly Conserved Novel 3' Terminal Sequence Element of the Hepatitis C Virus." However, the instant disclosure represents a comprehensive and detailed analysis of these conserved regions to the extent that permitted the discovery and evaluation of multiple short segments suitable for use alone and in

combination as a therapeutic for silencing HCV among a diverse patient population. The conserved sequences were screened against Genbank sequences of both human genomic and cDNA libraries (human chromosomes database), and the series of permuted HCV segments greater than 20 bases long with no homology to the human sequence databases were identified.

Replace the paragraph beginning at page 13, line 12, with the following amended paragraph:

Since the length of a contiguous dsRNA sequence capable of association with and activation of RISC (RNA-induced silencing complex), is generally considered to be 19-27 base pairs, the identified conserved HBV and HCV sequences were compared with both human genomic libraries and, perhaps even more importantly, with human cDNA libraries as described above. Since human cDNA libraries represent expressed sequences that appear in mRNAs, such mRNA sequences would be especially vulnerable to silencing by homologous dsRNA sequences provided to a cell.

Replace the paragraph beginning at page 15, line 4, with the following amended paragraph:

HBV Conserved Region 5

[C(69%)/del(31%)][G(69%)/del(31%)]A[G(85%)/T(11%)/C(4%)]GCAGG
TCCCCTAGAAGAAGAACTCCCTGCCTCGCAGACG[C(61%)/A(39%)]AG[A(62%)/
G(38%)]TCTCAATCG[C(88%)/A(12%)]CGCGTCGCAGAAGATCTCAAT[C(92%)/T(8
%)]TCGGGAATCT[C(88%)/T(12%)]AATGTTAGTAT

Replace the section beginning at page 17, line 19, and ending at page 18, line 5, with the following amended section:

SEQ ID NO:5 HBV

CGAbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCCTGCAGACG
mAGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTA
GTAT

SEQ ID NO:6 HBV

AbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCCTGCAGACGmA
GrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTAGTA
T

SEQ ID NO:7 HBV

CAbGCAGGTCCCCTAGAAGAAGAAACTCCCTGCCCTGCAGACGm
AGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTAGT
AT

SEQ ID NO:8 HBV

GAbGCAGGTCCCCTAGAAGAAGAAACTCCCTGCCCTGCAGACGm
AGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTAGT
AT

Replace the section beginning at page 19, line 25, and ending at page 24, line 21, with the following amended section:

By "dsRNA" or "dsRNA effector molecule" is meant a nucleic acid containing a region of two or more nucleotides that are in a double stranded conformation. It is envisioned that the conserved viral sequences of the invention may be utilized in any of the many compositions of "dsRNA effector molecules" known in the art or subsequently developed which act through a dsRNA-mediated gene silencing or RNAi mechanism, including, e.g., "hairpin" or stem-loop double-stranded RNA effector molecules in which a single RNA strand with self-complementary sequences is capable of assuming a double-stranded conformation, or duplex dsRNA effector molecules comprising two separate strands of RNA,. In various embodiments, the dsRNA consists entirely of ribonucleotides or consists of a mixture of ribonucleotides

and deoxynucleotides, such as the RNA/DNA hybrids disclosed, for example, by WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. The dsRNA or dsRNA effector molecule may be a single molecule with a region of self-complementarity such that nucleotides in one segment of the molecule base pair with nucleotides in another segment of the molecule. In various embodiments, a dsRNA that consists of a single molecule consists entirely of ribonucleotides or includes a region of ribonucleotides that is complementary to a region of deoxyribonucleotides. Alternatively, the dsRNA may include two different strands that have a region of complementarity to each other. In various embodiments, both strands consist entirely of ribonucleotides, one strand consists entirely of ribonucleotides and one strand consists entirely of deoxyribonucleotides, or one or both strands contain a mixture of ribonucleotides and deoxyribonucleotides.

Desirably, the regions of complementarity are at least 70, 80, 90, 95, 98, or 100% complementary to each other and to a target nucleic acid sequence. Desirably, the region of the dsRNA that is present in a double stranded conformation includes at least 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 50, 75, 100, 200, 500, 1000, 2000 or 5000 nucleotides or includes all of the nucleotides in a cDNA or other target nucleic acid sequence being represented in the dsRNA. In some embodiments, the dsRNA does not contain any single stranded regions, such as single stranded ends, or the dsRNA is a hairpin. In other embodiments, the dsRNA has one or more single stranded regions or overhangs. Desirable RNA/DNA hybrids include a DNA strand or region that is an antisense strand or region (e.g., has at least 70, 80, 90, 95, 98, or 100% complementarity to a target nucleic acid) and an RNA strand or region that is a sense strand or region (e.g., has at least 70, 80, 90, 95, 98, or 100% identity to a target nucleic acid), and vice versa. In various embodiments, the RNA/DNA hybrid is made *in vitro* using enzymatic or chemical synthetic methods such as those described herein or those described in WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. In other embodiments, a DNA strand synthesized *in vitro* is complexed with an RNA strand made *in vivo* or *in vitro* before, after, or concurrent with the transformation of the DNA strand into the cell. In yet other embodiments, the dsRNA is a single circular nucleic acid containing a sense and an antisense region, or the dsRNA includes a circular nucleic acid and either a second circular nucleic acid or a linear nucleic acid (see, for example, WO 00/63364,

filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999.) Exemplary circular nucleic acids include lariat structures in which the free 5' phosphoryl group of a nucleotide becomes linked to the 2' hydroxyl group of another nucleotide in a loop back fashion.

In other embodiments, the dsRNA includes one or more modified nucleotides in which the 2' position in the sugar contains a halogen (such as fluorine group) or contains an alkoxy group (such as a methoxy group) which increases the half-life of the dsRNA *in vitro* or *in vivo* compared to the corresponding dsRNA in which the corresponding 2' position contains a hydrogen or an hydroxyl group. In yet other embodiments, the dsRNA includes one or more linkages between adjacent nucleotides other than a naturally-occurring phosphodiester linkage. Examples of such linkages include phosphoramido, phosphorothioate, and phosphorodithioate linkages. The dsRNAs may also be chemically modified nucleic acid molecules as taught in U.S. Patent No. 6,673,661. In other embodiments, the dsRNA contains one or two capped strands, as disclosed, for example, by WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. In other embodiments, the dsRNA contains coding sequence or non-coding sequence, for example, a regulatory sequence (e.g., a transcription factor binding site, a promoter, or a 5' or 3' untranslated region (UTR) of an mRNA). Additionally, the dsRNA can be any of the at least partially dsRNA molecules disclosed in WO 00/63364, filed April 19, 2000 (see, for example, pages 8-22), as well as any of the dsRNA molecules described in US Provisional Application 60/399,998 filed July 31, 2002, and PCT/US2003/024028, filed 31-Jul-2003; and US Provisional Application 60/419,532 filed October 18, 2002, and PCT/US2003/033466, filed 20-Oct-2003, the teaching of which is hereby incorporated by reference. Any of the dsRNAs may be expressed *in vitro* or *in vivo* using the methods described herein or standard methods, such as those described in WO 00/63364, filed April 19, 2000 (see, for example, pages 16-22). In some preferred embodiments, multiple anti-HBV and/or anti-HCV dsRNA effector molecules of the invention are transcribed in a mammalian cell from one or more expression constructs each comprising multiple polymerase III promoter expression cassettes as described in more detail in US 60/603622; US 60/629942; and PCT/US05/29976 filed 23-August-2005; "Multiple Polymerase III Promoter Expression Constructs"; the teaching of which is incorporated by reference.

dsRNA "Hairpin" Constructs or dsRNA "Hairpin" Expression Vectors:

Constructs encoding a unimolecular hairpin dsRNA are more desirable for some applications than constructs encoding duplex dsRNA (i.e., dsRNA composed of one RNA molecule with a sense region and a separate RNA molecule with an antisense region) because the single-stranded RNA with inverted repeat sequences more efficiently forms a dsRNA hairpin structure. This greater efficiency is due in part to the occurrence of transcriptional interference arising in vectors containing converging promoters that generate duplex dsRNA. Transcriptional interference results in the incomplete synthesis of each RNA strand thereby reducing the number of complete sense and antisense strands that can base-pair with each other and form duplexes. Transcriptional interference can be overcome, if desired, through the use of (i) a two vector system in which one vector encodes the sense RNA and the second vector encodes the antisense RNA, (ii) a bicistronic vector in which the individual strands are encoded by the same plasmid but through the use of separate cistrons, or (iii) a single promoter vector that encodes a hairpin dsRNA, i.e., an RNA in which the sense and antisense sequences are encoded within the same RNA molecule.

Hairpin-expressing vectors have some advantages relative to the duplex vectors. For example, in vectors that encode a duplex RNA, the RNA strands need to find and base-pair with their complementary counterparts soon after transcription. If this hybridization does not happen, the individual RNA strands diffuse away from the transcription template and the local concentration of sense strands with respect to antisense strands is decreased. This effect is greater for RNA that is transcribed intracellularly compared to RNA transcribed *in vitro* due to the lower levels of template per cell. Moreover, RNA folds by nearest neighbor rules, resulting in RNA molecules that are folded co-transcriptionally (i.e., folded as they are transcribed). Some percentage of completed RNA transcripts is therefore unavailable for base-pairing with a complementary second RNA because of intra-molecular base-pairing in these molecules. The percentage of such unavailable molecules increases with time following their transcription. These molecules may never form a duplex because they are already in a stably folded structure. In a hairpin RNA, an RNA sequence is always in close physical proximity to its complementary RNA. Since RNA structure is not static, as the RNA transiently unfolds, its complementary sequence is immediately available and can participate in base-pairing because it is

so close. Once formed, the hairpin structure is predicted to be more stable than the original non-hairpin structure. Especially desirable are, e.g., "forced" hairpin constructs, partial hairpins capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002; and PCT/US2003/024028, "Double Stranded RNA Structures and Constructs and Methods for Generating and Using the Same," filed 31-Jul-2003; as well as the "udderly" structured hairpins, hairpins with mismatched regions, and multiepitope constructs as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, "Double-Stranded RNA Structures and Constructs, and Methods for Generating and Using the Same," filed 20-Oct-2003.

By "short dsRNA" is meant a dsRNA that has about 200, 100, 75, 50, 45, 40, 35, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20 or 19 contiguous nucleotides in length that are in a double stranded conformation. Desirably, the short dsRNA comprises a double-stranded region of at least 19 contiguous basepairs in length identical/complementary to a target sequence to be inhibited. In desirable embodiments, the double stranded region is between 19 to 50, 19 to 40, 19 to 30, 19 to 25, 20 to 25, 21 to 23, 25 to 30, or 30 to 40 contiguous basepairs in length, inclusive. In some embodiments, the short dsRNA is between 30 to 50, 50 to 100, 100 to 200, 200 to 300, 400 to 500, 500 to 700, 700 to 1000, 1000 to 2000, or 2000 to 5000 nucleotides in length, inclusive and has a double stranded region that is between 38 and 60 contiguous basepairs in length, inclusive. In one embodiment, the short dsRNA is completely double stranded. In some embodiments, the short dsRNA is between 11 and 30 nucleotides in length, and the entire dsRNA is double stranded. In other embodiments, the short dsRNA has one or two single stranded regions. In some embodiments, the short dsRNA is a "shRNA" or "short-hairpin RNA" or "shRNA effector molecule" or "dsRNA hairpin", meaning an RNA molecule of less than approximately 400 to 500 nucleotides (nt) in length, preferably less than 100 to 200 nt in length, in which at least one stretch of at least about 15 to 100 nucleotides (preferably 17 to 50 nt; more preferably 19 to 29 nt) is base paired with a complementary sequence located on the same RNA molecule, and where said sequence and complementary sequence are separated by an unpaired region of at least about 4 to 7 nucleotides (preferably about 9 to about 15 nucleotides) which forms a single-stranded loop above the stem structure created by the two regions of

base complementarity. The shRNA molecules comprise at least one stem-loop structure comprising a double-stranded stem region of about 17 to about 100 bp; about 17 to about 50 bp; about 40 to about 100 bp; about 18 to about 40 bp; or from about 19 to about 29 bp; homologous and complementary to a target sequence to be inhibited; and an unpaired loop region of at least about 4 to 7 nucleotides; preferably about 9 to about 15 nucleotides, which forms a single-stranded loop above the stem structure created by the two regions of base complementarity. Included shRNAs are dual or bi-finger (i.e., having two stem-loop structures) and multi-finger hairpin dsRNAs (having multiple stem-loop structures), in which the RNA molecule comprises two or more of such stem-loop structures separated by single-stranded spacer regions. In some embodiments, an expression construct may be used to express one or more of such shRNA molecules in a mammalian cell, including multiple copies of the same, and/or one or more, including multiple different, short hairpin RNA molecules. Short hairpin RNA molecules considered to be the "same" as each other are those that comprise only the same double-stranded sequence, and short hairpin RNA molecules considered to be "different" from each other will comprise different double-stranded sequences, regardless of whether the sequences to be targeted by each different double-stranded sequence are within the same, or a different gene, such as, e.g., sequences of a promoter region and of a transcribed region (mRNA) of the same gene, or sequences of two different genes.

In particular embodiments, the short dsRNA binds PKR or another protein in a dsRNA-mediated stress response pathway. Desirably, such a short dsRNA inhibits the dimerization and activation of PKR by at least 20, 40, 60, 80, 90, or 100%. In some desirable embodiments, the short dsRNA inhibits the binding of a long dsRNA to PKR or another component of a dsRNA-mediated stress response pathway by at least 20, 40, 60, 80, 90, or 100%. See also the teaching of USSN 10/425,006, filed 28-Apr-2003, "Methods of Silencing Genes Without Inducing Toxicity", Pachuk, as to utilization of short dsRNAs in conjunction with other dsRNAs to avoid dsRNA-mediated toxicity. The applicants have demonstrated, however, that dsRNA molecules, even long dsRNA molecules, are in general unlikely to evoke a significant dsRNA stress response, including a PKR or interferon or "panic" response, if they are expressed intracellularly in the mammalian (or other vertebrate) cell in which the RNAi effect is desired. See, e.g., US 2002/0132257, "Use of post-transcriptional

gene silencing for identifying nucleic acid sequences that modulate the function of a cell". Accordingly, such "expressed interfering RNA molecules" or "eiRNA" molecules and "eiRNA expression constructs", i.e., dsRNA molecules (or the corresponding dsRNA expression constructs) expressed intracellularly or endogenously in vivo within the mammalian cell in which dsRNA gene silencing or RNAi is induced, are preferred in some aspects of the invention.

By "at least 19 contiguous base pair nucleotide sequence" is meant that a nucleotide sequence can start at any nucleotide within one of the disclosed sequences, so long as the start site is capable of producing a polynucleotide of at least 19 contiguous base pairs. For example, an at least 19 contiguous base pair nucleotide sequence can comprise nucleotide 1 through nucleotide 19, nucleotide 2 through nucleotide 20, nucleotide 3 through nucleotide 21, and so forth to produce a 19mer. Thus, a 20mer can comprise nucleotide 1 through nucleotide 20, nucleotide 2 through nucleotide 21, nucleotide 3 through nucleotide 22, and so forth. Similar sequences above 20 contiguous nucleotides, e.g., 21, 22, 23, 24, 25, 26, 27, etc. selected from within the conserved sequences are envisioned. Such a sequence of at least 19 contiguous nucleotides (in double-stranded conformation with its complement) is "an at least 19 contiguous base pair sequence" and may be present as a duplex dsRNA, within a dsRNA hairpin, or encoded in a dsRNA expression construct.

Replace the section beginning at page 25, line 3, and ending at page 26, line 16, with the following amended section:

By an "expression construct" is meant any double-stranded DNA or double-stranded RNA designed to transcribe an RNA, e.g., a construct that contains at least one promoter operably linked to a downstream gene or coding region of interest (e.g., a cDNA or genomic DNA fragment that encodes a protein, or any RNA of interest). Transfection or transformation of the expression construct into a recipient cell allows the cell to express RNA or protein encoded by the expression construct. An expression construct may be a genetically engineered plasmid, virus, or artificial chromosome derived from, for example, a bacteriophage, adenovirus, retrovirus, poxvirus, or herpesvirus. An expression construct does not have to be replicable in a

living cell, but may be made synthetically. An expression construct or expression vector engineered to express a double-stranded RNA effector molecule or dsRNA molecule is a "dsRNA expression construct" or "dsRNA expression vector".

In one embodiment of the invention, a recombinant expression vector or expression construct is engineered to express multiple, e.g., three, four, five or more short hairpin dsRNA effector molecules, each expressed from a different expression cassette comprising a polymerase III promoter, one or more, including all of which, may be different from the others. In one aspect of the invention, a recombinant expression vector transcribing three, four, five or more different shRNA molecules (each comprising a double-stranded "stem" region comprising at least 19 contiguous basepairs from/complementary to a conserved HBV and/or HCV sequence) is used to inhibit replication of hepatitis B virus (HBV) and/or hepatitis C virus (HCV). In one embodiment, each shRNA molecule is expressed under the control of a polymerase III promoter, e.g., 7SK, H1, and U6, which may be the same or different. Such dsRNA expression constructs comprising multiple polymerase III expression cassettes are described in greater detail in PCT/US05/29976, "Multiple Polymerase III Promoter Expression Constructs", the teaching of which is hereby incorporated by reference. In one aspect, a recombinant expression vector or expression construct of the invention may express one or more bi-fingered or multi-fingered dsRNA hairpin molecules from one or more polymerase III promoter-driven transcription units as well as one or more single hairpin dsRNA molecules from one or more polymerase III promoter-driven transcription units. It will be understood that in any of said expression constructs transcribing a hairpin dsRNA from a polymerase III promoter, the hairpin dsRNA may be a single hairpin dsRNA or a bi-fingered, or multi-fingered dsRNA hairpin as described in WO2004/035765, published 29-Apr-2004, or a partial or forced hairpin structure as described in WO2004/011624, published 5-Feb-2004, the teaching of which is incorporated herein by reference.

By "operably linked" is meant that a nucleic acid sequence or molecule and one or more regulatory sequences (e.g., a promoter, enhancer, repressor, terminator) are connected in such a way as to permit transcription of an RNA molecule, e.g., a single-stranded RNA molecule such as a sense, antisense, a dsRNA hairpin, or an mRNA, or permit expression and translation and/or secretion of

the product (i.e., a polypeptide) of the nucleic acid molecule when the appropriate molecules are bound to the regulatory sequences.

By a "promoter" is meant a nucleic acid sequence sufficient to direct transcription of a covalently linked nucleic acid molecule. Also included in this definition are those transcription control elements (e.g., enhancers) that are sufficient to render promoter-dependent gene expression controllable in a cell type-specific, tissue-specific, or temporal-specific manner, or that are inducible by external signals or agents; such elements, which are well-known to skilled artisans, may be found in a 5' or 3' region of a gene or within an intron. See, e.g., published U.S. Patent Application No. 2005/0130184 A1, 16-June-2005, Xu et al., directed to modified polymerase III promoters which utilize polymerase II enhancer elements, as well as Published U.S. Patent Application No. 2005/0130919 A1, 16-June-2005, Xu et al., directed to regulatable polymerase III and polymerase II promoters, the teaching of which is hereby incorporated by reference. Desirably a promoter is operably linked to a nucleic acid sequence, for example, a cDNA or a gene sequence, or a sequence encoding a dsRNA, e.g., a shRNA, in such a way as to permit expression of the nucleic acid sequence.

The RNA molecule according to this invention may be delivered to the mammalian cell or extracellular pathogen present in the mammalian cell in the composition as a dsRNA effector molecule or partially double stranded RNA sequence, or RNA/DNA hybrid, which was made *in vitro* by conventional enzymatic synthetic methods using, for example, the bacteriophage T7, T3 or SP6 RNA polymerases according to the conventional methods described by such texts as the Promega Protocols and Applications Guide, (3rd ed. 1996), eds. Doyle, ISBN No. 1 57 Alternatively these molecules may be made by chemical synthetic methods *in vitro* [see, e.g., Q. Xu et al., Nucleic Acids Res., 24(18):3643-4 (Sept. 1996); N. Naryshkin et al., Bioorg. Khim., 22(9):691-8 (Sept. 1996); J. A. Grasby et al., Nucleic Acids Res., 21(19):4444-50 (Sept 1993); C. Chaix et al., Nucleic Acids Res. 17:7381-93 (1989); S.H. Chou et al., Biochem., 28(6):2422-35 (Mar. 1989); O. Odal el al., Nucleic Acids Symp. Ser., 21:105-6 (1989); N.A. Naryshkin et al., Bioorg. Khim, 22(9):691-8 (Sept. 1996); S. Sun et al., RNA, 3(11):1352-1363 (Nov. 1997); X. Zhang et al., Nucleic Acids Res., 25(20):3980-3 (Oct. 1997); S. M. Grvaznov el al., Nucleic Acids Res., 2-6 (18):4160-7 (Sept. 1998); M. Kadokura et al., Nucleic Acids

Symp. Ser., 37:77-8 (1997); A. Davison et al., Biomed. Pept. Proteins. Nucleic Acids, 2(I):1-6 (1996); and A. V. Mudrakovskaia et al., Bioorg. Khim., 17(6):819-22 (Jun. 1991)].

Replace the paragraph beginning at page 27, line 6, with the following amended paragraph:

Preferably the single stranded RNA sense or anti-sense strand forms a hairpin at one or both termini. Desirably, the single stranded RNA sense or anti-sense strand forms a hairpin at some intermediate portion between the termini. Such a single stranded RNA sense or anti-sense strand may also be designed to fold back upon itself to become partially double stranded *in vitro* or *in vivo*. Yet another embodiment of an extant RNA molecule as the effective agent used in the compositions is a single stranded RNA sequence comprising both a sense polynucleotide sequence and an antisense polynucleotide sequence, optionally separated by a non-base paired polynucleotide sequence. Preferably, this single stranded RNA sequence has the ability to become double-stranded once it is in the cell, or *in vitro* during its synthesis. In desirable embodiments, a sequence of at least about 19 to 29 contiguous basepairs will assume a double-stranded conformation. In desirable embodiments, the double-stranded region will include an at least about 19 contiguous basepair sequence identical/complementary to a target nucleotide sequence to be downregulated or inhibited.

Replace the section beginning at page 28, line 3, and ending at page 29, line 27, with the following amended section:

Alternatively, the RNA molecule may be formed *in vivo* and thus delivered by a "delivery agent" which generates such a partially double-stranded RNA molecule *in vivo* after delivery of the agent to the mammalian cell or to the mammal. Thus, the agent which forms the composition of this invention is, in one embodiment, a double stranded DNA molecule "encoding" one of the above-described RNA molecules, e.g., a dsRNA expression vector or expression construct. The DNA agent provides the nucleotide sequence which is transcribed within the cell to become a double

stranded RNA. In another embodiment, the DNA sequence provides a deoxyribonucleotide sequence which within the cell is transcribed into the above-described single stranded RNA sense or anti-sense strand, which optionally forms a hairpin at one or both termini or folds back upon itself to become partially double stranded. The DNA molecule which is the delivery agent of the composition can provide a single stranded RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide sequence, optionally separated by a nonbase paired polynucleotide sequence, and wherein the single stranded RNA sequence has the ability to become double-stranded. Alternatively, the DNA molecule which is the delivery agent provides for the transcription of the above-described circular RNA molecule that optionally forms a rod structure or partial double strand *in vivo*. The DNA molecule may also provide for the *in vivo* production of an RNA/DNA hybrid as described above, or a duplex containing one RNA strand and one DNA strand. These various DNA molecules may be designed by resort to conventional techniques such as those described in Sambrook, cited above or in the Promega reference, cited above.

A latter delivery agent of the present invention, which enables the formation in the mammalian cell of any of the above-described RNA molecules, can be a DNA single stranded or double stranded plasmid or vector. Expression vectors designed to produce RNAs as described herein *in vitro* or *in vivo* may contain sequences under the control of any RNA polymerase, including mitochondrial RNA polymerase, RNA pol I, RNA pol II, and RNA pol III, and viral polymerases, and bacteriophage polymerases such as T7 and Sp6. Desirably, expression vectors designed for *in vivo* expression of dsRNA effector molecules within a mammalian cell may be designed to utilize an endogenous mammalian polymerase such as an RNA polymerase I, RNA polymerase II, RNA polymerase III, and/or a mitochondrial polymerase. Expression vectors utilizing cognate promoter(s), e.g., polymerase III promoters such as U6, H1, or 7SK, in order to effect transcription by RNA polymerase III can readily be designed. Preferred for expression of short RNA molecules less than about 400 to 500 nucleotides in length are RNA polymerase III promoters. In some aspects, an "RNA polymerase III promoter" or "RNA pol III promoter" or "polymerase III promoter" or "pol III promoter" is preferred, meaning any invertebrate, vertebrate, or mammalian promoter, e.g., human, murine, porcine, bovine, primate, simian, etc.

that, in its native context in a cell, associates or interacts with RNA polymerase III to transcribe its operably linked gene, or any variant thereof, natural or engineered, that will interact in a selected host cell with an RNA polymerase III to transcribe an operably linked nucleic acid sequence. Preferred in some applications are the Type III RNA pol III promoters including U6, H1, 7SK, and MRP which exist in the 5' flanking region, include TATA boxes, and lack internal promoter sequences. One reason RNA Pol III promoters are especially desirable for expression of small engineered RNA transcripts is that RNA Pol III termination, unlike RNA polymerase II termination, occurs efficiently and precisely at a short run of thymine residues in the DNA coding strand, without other protein factors, T₄ and T₅ being the shortest Pol III termination signals in yeast and mammals, with oligo (dT) terminators longer than T₅ being very rare in mammals. Accordingly, the multiple polymerase III promoter expression constructs of the invention will include an appropriate oligo (dT) termination signal, i.e., a sequence of 4, 5, 6 or more Ts, operably linked 3' to each RNA Pol III promoter in the DNA coding strand.

These vectors can be used to transcribe the desired RNA molecule in the cell according to this invention. Vectors may be desirably designed to utilize an endogenous mitochondrial RNA polymerase (e.g., human mitochondrial RNA polymerase, in which case such vectors may utilize the corresponding human mitochondrial promoter). Mitochondrial polymerases may be used to generate capped (through expression of a capping enzyme) or uncapped messages *in vivo*. RNA pol I, RNA pol II, and RNA pol III transcripts may also be generated *in vivo*. Such RNAs may be capped or not, and if desired, cytoplasmic capping may be accomplished by various means including use of a capping enzyme such as a vaccinia capping enzyme or an alphavirus capping enzyme. However, all pol II transcripts are capped. The DNA vector is designed to contain one of the promoters or multiple promoters in combination (mitochondrial, RNA pol I, pol II, or pol III, or viral, bacterial or bacteriophage promoters along with the cognate polymerases). Preferably, where the promoter is RNA pol II, the sequence encoding the RNA molecule has an open reading frame greater than about 300 nts and must follow the rules of design to prevent nonsense-mediated degradation in the nucleus. Such plasmids or vectors can include plasmid sequences from bacteria, viruses or phages.

Such vectors include chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses, vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids and phagemids.

Replace the section beginning at page 31, line 26, and ending at page 34, line 29, with the following amended section:

The term "*in vivo*" is intended to include any system wherein the cellular DNA or RNA replication machinery is intact, preferably within intact living cells, including tissue culture systems, tissue explants, and within single cell or multicellular living organisms.

By "multiple sequitope dsRNA" or "multisequitope dsRNA" or "multiple epitope dsRNA" is meant an RNA molecule that has segments derived from multiple target nucleic acids or that has non-contiguous segments from the same target nucleic acid. For example, the multiple sequitope dsRNA may have segments derived from (i) sequences representing multiple genes of a single organism; (ii) sequences representing one or more genes from a variety of different organisms; and/or (iii) sequences representing different regions of a particular gene (e.g., one or more sequences from a promoter and one or more sequences from an mRNA. Desirably, each segment has substantial sequence identity to the corresponding region of a target nucleic acid. In various desirable embodiments, a segment with substantial sequence identity to the target nucleic acid is at least 19, 20, 21, 22, 23, 24, 25, 26, 27, 30, 40, 50, 100, 200, 500, 750, or more basepairs in length. In desirable embodiments, the multiple epitope dsRNA inhibits the expression of at least 2, 4, 6, 8, 10, 15, 20, or more target genes by at least 20, 40, 60, 80, 90, 95, or 100%. In some embodiments, the multiple epitope dsRNA has non-contiguous segments from the same target gene or from the same target polynucleotide that may or may not be in the naturally occurring 5' to 3' order of the segments, and the dsRNA inhibits the expression of the target nucleic acid by at least 50, 100, 200, 500, or 1000% more than a dsRNA with only one of the segments.

By "sequitope" is meant a contiguous sequence of double-stranded polyribonucleotides that can associate with and activate RISC (RNA-induced silencing complex), usually a contiguous sequence of between 19 and 27 basepairs, inclusive. Sequences comprising at least one sequitope from within one or more of the conserved HBV and/or HCV nucleotide sequences identified here may be utilized for dsRNA mediated gene silencing as taught herein.

Multiple-epitope/ multiple-sequitope dsRNAs the advantages of a multiple-epitope or multisequitope double-stranded RNA approach as taught in USSN 60/419,532, filed 18-Oct-2002 and PCT/US2003/033466, filed 20-Oct-2003, are applicable to utilization of the conserved HBV and/or HCV sequences of the invention. Because a singular species of dsRNA can simultaneously silence many target genes (e.g., genes from multiple pathogens, multiple genes or sequences from a single pathogen, or genes associated with multiple diseases), a multiple epitope dsRNA can be used for many different indications in the same subject or used for a subset of indications in one subject and another subset of indications in another subject. For such applications, the ability to express long dsRNA molecules (e.g., dsRNA molecules with sequences from multiple genes) without invoking the dsRNA stress response is highly desirable. For example, by using a series of sequences, each, e.g., as short as 19-21 nucleotides, desirably 100 to 600 nucleotides, or easily up to 1, 2, 3, 4, 5, or more kilobases such that the total length of such sequences is within the maximum capacity of the selected plasmid (e.g., 20 kilobases in length), a single such pharmaceutical composition can provide protection against a large number of pathogens and/or toxins at a relatively low cost and low toxicity, e.g., HBV, HCV, HIV, etc.

The use of multiple epitopes or sequitopes derived from one or more genes and/or different overlapping and/or non-contiguous sequences of the same polynucleotide or gene from multiple strains and/or variants of a highly variable or rapidly mutating pathogen such as HBV and/or HCV can also be very advantageous. For example, a singular dsRNA species that recognizes and targets multiple strains and/or variants of HBV and/or HCV can be used as a universal treatment or vaccine for the various strains/variants of HBV and/or HCV.

The ability to silence multiple genes of a particular pathogen such as HBV and/or HCV prevents the selection of, in this case, HBV and/or HCV "escape

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mutants." In contrast, typical small molecule treatment or vaccine therapy that only targets one gene or protein results in the selection of pathogens that have sustained mutations in the target gene or protein and the pathogen thus becomes resistant to the therapy. By simultaneously targeting a number of genes or sequences of the pathogen and/or extensive regions of the pathogen using the multiple epitope approach of the present invention, the emergence of such "escape mutants" is effectively precluded.

For example, it is considered particularly advantageous to include a mixture of sequences from both HCV SEQ ID NO:11 and SEQ ID NO:12, and SEQ ID NO: 27, i.e., one or more sequences (e.g, each at least 19, 20, 21, 22, 23, 24, 25, 26, 27 to 29 contiguous nucleotides) from HCV SEQ ID NO:11 together with one or more sequences (e.g, each at least 19, 20, 21, 22, 23, 24, 25, 26, 27 to 29 contiguous nucleotides) from HCV SEQ ID NO:12 and from SEQ ID NO: 27 , either in a single dsRNA molecule, an admixture of dsRNA molecules, or through concomitant administration of such molecules to a patient (or by administering one or more dsRNA expression constructs which produce such dsRNA molecules intracellularly), in order to decrease the ability of the virus to generate viable escape mutants. Similarly, it would be advantageous to provide a mixture of dsRNA molecules comprising a number of the conserved HBV sequences, in some cases in combination with one or more of the conserved HCV sequences of the invention.

Similarly, it may be desirable to use sequences from two or more of HBV SEQ ID NO:1, SEQ ID NO:2, AND SEQ ID NO:3, either in a single dsRNA construct, an admixture of constructs, or through concomitant administration of such constructs (or dsRNA expression constructs which produce such dsRNA molecules) to a patient. SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 map to the HBV surface antigen genes. Due to the overlapping nature of the HBV mRNAs, the following mRNAs would be targeted by one of more of these sequences: Surface Ag (sAg) mRNAs, precore, core and polymerase mRNAs. However, since sAg mRNAs are the most abundant, it is more likely that these mRNAs will be targeted if the gene-silencing machinery is saturable. It is possible, however, that all listed mRNAs will be targeted. Reduction of surface Ag is desirable for several reasons: a) surface Ag is needed for assembly of infectious virions; b) overexpression of Surface Ag during infection is thought to contribute to immune anergy that occurs during chronic HBV infection;

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and c) the expression of HBVsAg in the livers of infected individuals (even in the absence of virus, i.e., from integrated sAg sequences into the host genome) induces hepatitis. Therefore, reduction of sAg is likely to decrease viral titers, overcome immune anergy and decrease/prevent hepatitis.

Replace the section beginning at page 35, line 3, and ending at page 42, line 2, with the following amended section:

HBV SEQ ID NO:5 through SEQ ID NO:8 map to the polymerase gene. Effector RNAs are predicted to target only precore/core and polymerase transcripts. There should be no competition with sAg or X mRNAs. Polymerase is needed for the synthesis of viral genomes and therefore viral particle titer is expected to decrease as polymerase is decreased.

HBV SEQ ID NO:9 maps to the X gene. Due to the terminal redundancy of all the HBV mRNAs, these effector RNAs have the potential to target all of the HBV viral mRNAs. X protein has many ascribed (non proven) functions. Evidence is emerging, however, that X-gene expression is associated with hepatocellular carcinogenesis, in part related to promotion of detachment and migration of cells out of the primary tumor site. Since the X gene is often found in integrated HBV sequences in individuals with and without active hepatitis, down-regulation of X gene expression is predicted to ameliorate disease, including the incidence of hepatocellular carcinoma.

In general, the more sequences or sequitopes from the different identified sequences that are used (e.g., from SEQ ID NO:1, SEQ ID NO:2, and/or SEQ ID NO:3, plus sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10), the less likely a virus will be able to generate viable escape mutants. Also, the more different mRNAs that can be targeted, the more significant will be the drops in viral titer and disease amelioration.

Desirable combinations for multiepitope or multisequitope dsRNA expression constructs or dsRNA effector molecules, an admixture of dsRNA expression constructs or dsRNA effector molecules , or the concomitant administration of different dsRNA expression constructs or dsRNA effector molecules include the following: Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus

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sequences from SEQ ID NO:4; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:5; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:10;

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NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9 and SEQ ID NO:10; Sequences from SEQ ID NO:4 and SEQ ID NO:5; Sequences from SEQ ID NO:4 and SEQ ID NO:6; Sequences from SEQ ID NO:4 and SEQ ID NO:7; Sequences from SEQ ID NO:4 and SEQ ID NO:8; Sequences from SEQ ID NO:4 and SEQ ID NO:9; Sequences from SEQ ID NO:4 and SEQ ID NO:10; Sequences from SEQ ID NO:5 and SEQ ID NO:6; Sequences from SEQ ID NO:5 and SEQ ID NO:7; Sequences from SEQ ID NO:5 and SEQ ID NO:8; Sequences from SEQ ID NO:5 and SEQ ID NO:9; Sequences from SEQ ID NO:5 and SEQ ID NO:10; Sequences from SEQ ID NO:6 and SEQ ID NO:7; Sequences from SEQ ID NO:6 and SEQ ID NO:8; Sequences from SEQ ID NO:6 and SEQ ID NO:9; Sequences from SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:7 and SEQ ID NO:9; Sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences from SEQ ID NO:9 and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6; Sequences from SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:7, and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:7, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7; Sequences from SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:8;

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Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:5, SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:6, SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID NO:6, SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:6, SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:7, SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:7, SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:8, SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:6; and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:6; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:7; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:9; Sequences from SEQ ID NO:5,

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SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:7; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:6, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9;

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Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; and Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10. Preferred in some aspects are sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8, including combinations of sequitopes from SEQ ID NO:5, plus SEQ ID NO:6, plus SEQ ID NO:7, plus SEQ ID NO:8.

In another embodiment, combinations of sequitopes at least 19 contiguous base pairs in length and longer sequences from within any of the aforementioned sequences (e.g., SEQ ID NO:1 through SEQ ID NO:12) may be utilized either in a single dsRNA expression construct or dsRNA effector molecule, an admixture of dsRNA expression constructs or dsRNA effector molecules or through concomitant administration of such dsRNA expression constructs or dsRNA effector molecules to a patient. By a sequence of "at least 19 contiguous base pairs in length" is meant that a sequence or sequitope of at 19 contiguous bases in length is present in double-stranded conformation, or within a double-stranded RNA effector molecule.

As discussed elsewhere herein, a particularly preferred embodiment of the invention utilizes dsRNA expression constructs or vectors to achieve endogenous delivery of the dsRNAs of the invention, especially the multiple different sequences described above. These dsRNAs may be provided e.g., on the same cistron of an expression construct such as a plasmid, on different cistrons of an expression construct, or on different expression constructs or plasmids, e.g., one or more plasmids and/or one or more vectors, including viral vectors. The combination of different dsRNA effector molecules such as shRNA effector molecules may be provided to a mammalian cell by *in vivo* expression from a single expression construct such as a plasmid, with each dsRNA effector molecule transcribed from a different expression cassette driven by a different promoter, e.g., an RNA

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polymerase I promoter and/or an RNA polymerase III promoter, e.g., a type 3 RNA polymerase III promoter such as U6, H1, 7SK, or MRP. In some embodiments, each such different expression cassette may contain a different RNA polymerase III promoter, which may be the same or different, and an RNA polymerase III termination sequence. In another embodiment, a combination of different dsRNA effector molecules such as shRNA effector molecules may be provided to a mammalian cell by in vivo expression from a single expression construct such as a plasmid or a viral vector which comprise an expression cassette comprising multiple different promoters, e.g., an RNA polymerase I promoter and/or an RNA polymerase III promoter, e.g., a type 3 RNA polymerase III promoter such as U6, H1, 7SK, or MRP, and wherein each of such promoters transcribes a different dsRNA effector molecule. Such multiple different dsRNA effector sequences may also be provided to an in vivo mammalian cell exogenously, in any different mixture of one or more dsRNA structures, duplexes and/or harpins, and/or in combination with one or more endogenously expressed dsRNA structures.

Desirable methods of administration of nucleic acids The DNA and/or RNA constructs, e.g., dsRNA effector molecules, of the invention may be administered to the host cell/tissue/organism as "naked" DNA, RNA, or DNA/RNA, formulated in a pharmaceutical vehicle without any transfection promoting agent. More efficient delivery may be achieved as known to those of skill in the art of DNA and RNA delivery, using e.g., such polynucleotide transfection facilitating agents known to those of skill in the art of RNA and/or DNA delivery. The following are exemplary agents: cationic amphiphiles including local anesthetics such as bupivacaine, cationic lipids, liposomes or lipidic particles, polycations such as polylysine, branched, three-dimensional polycations such as dendrimers, carbohydrates, detergents, or surfactants, including benzylammonium surfactants such as benzalkonium chloride. Non-exclusive examples of such facilitating agents or co-agents useful in this invention are described in U.S. Patent numbers 5,593,972; 5,703,055; 5,739,118; 5,837,533; 5,962,482; 6,127,170; 6,379,965; and 6,482,804; and International Patent Application No. PCT/US98/22841; the teaching of which is hereby incorporated by reference. U.S. Patents numbers 5,824,538; 5,643,771; and 5,877,159 (incorporated herein by reference) teach delivery of a composition other

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than a polynucleotide composition, e.g., a transfected donor cell or a bacterium containing the dsRNA-encoding compositions of the invention.

Replace the paragraph beginning at page 43, line 20, with the following amended paragraph:

In general, targeting for selective delivery of the dsRNA constructs of the invention to hepatocytes is preferred. Targeting to hepatocytes may be achieved by coupling to ligands for hepatocyte-specific receptors. For example, asialo-orosomucoid, (poly)L-lysine-asialo-orosomucoid, or any other ligands of the hepatic asialoglycoprotein receptor (Spiess, Biochemistry 29(43):10009-10018, 1990; Wu et al., J. Biol. Chem. 267(18):12436-12439, 1992; Wu et al., Biotherapy 3:87-95, 1991). Similarly, the oligonucleotides may be targeted to hepatocytes by being conjugated to monoclonal antibodies that specifically bind to hepatocyte-specific receptors. Oligonucleotides may also be targeted to hepatocytes using specific vectors, as described below.

Replace the paragraph beginning at page 44, line 6, with the following amended paragraph:

The dsRNA oligonucleotides of the invention may be provided exogenously to a target hepatocyte, e.g., prepared outside the cell and delivered into a mammalian hepatocyte. Alternatively, a dsRNA may be produced within the target cell by transcription of a nucleic acid molecule comprising a promoter sequence operably linked to a sequence encoding the dsRNA. In this method, the nucleic acid molecule is contained within a non-replicating linear or circular DNA or RNA molecule, or is contained within an autonomously replicating plasmid or viral vector, or is integrated into the host genome. Any vector that can transfect a hepatocyte may be used in the methods of the invention. Preferred vectors are viral vectors, including those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses (see, e.g., WO89/07136; Rosenberg et al., N. Eng. J. Med. 323(9):570-578, 1990), adenovirus (see, e.g., Morsey et al., J. Cell. Biochem., Supp. 17E, 1993; Graham et al., in Murray, ed., Methods in Molecular Biology: Gene Transfer and Expression

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Protocols. Vol. 7, Clifton, N.J.: the Human Press 1991: 109-128), adeno-associated virus (Kotin et al., Proc. Natl. Acad. Sci. USA 87:2211-2215, 1990), replication defective herpes simplex viruses (HSV; Lu et al., Abstract, page 66, Abstracts of the Meeting on Gene Therapy, Sep. 22-26, 1992, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), and any modified versions of these vectors. Methods for constructing expression vectors are well known in the art (see, e.g., Molecular Cloning: A Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, N.Y., 1989).

Add, before the paragraph beginning at page 45, line 3, the following new section:

Upon assembly of a recombinant DNA plasmid dsRNA expression vector on the invention, bacteria are used as "factories" to produce large quantities of the final vector. The E.coli bacterium is frequently used for plasmid fermentation, and it may be advantageous to employ for this purpose E. coli strains having a reduced genome as described in, e.g., Blattner et al., Published U.S. Patent Application No. 2005/0032225, the teaching of which is incorporated herein by reference. The vector manufactured in this manner, isolated and purified according to methods known in the art, can be introduced into living cells with a variety of methods, collectively known as "transfection", including the methods and compositions described above. Once inside the cell, the promoter elements are recognized by the cellular machinery available for gene transcription and the RNA effector molecules, e.g., shRNAs, will be produced.

Other bacterial strains that may be advantageous for propagating a plasmid expression vector of the invention include the E. coli GT116 Competent Cells available commercially from InvivoGen, San Diego, CA. GT116 is a sbcCD deletion strain specifically engineered to support the growth of plasmid DNAs carrying hairpin structures, such as the plasmids of the invention engineered to express one or more dsRNA effector molecules which are hairpin RNAs. Hairpin structures are known to be unstable in E. coli due to their elimination by a protein complex called SbcCD that recognizes and cleaves hairpins (Connelly et al., Proc. Natl. Acad. Sci. USA 95:7969-74 (1998)). The sbcCD and sbcD genes are deleted in E. coli GT116,

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which improves its utility for cloning plasmids with hairpin or other palindrome-containing structures.

Replace the paragraph beginning at page 45, line 26, with the following amended paragraph:

dsRNA expression vectors may include promoters for RNA polymerase I, RNA polymerase II including but not limited to HCMV, SCMV, MCMV, RSV, EF2a, TK and other HSV promoters such as ICP6, ICP4 and ICP0 promoters, HBV pregenomic promoter, RNA pol III promoter, especially type 3 RNA polymerase III promoters, including but not limited to 7SK, U6, and H1, and tRNA promoters, as well as mitochondrial light and heavy strand promoters. Desirably, the dsRNA expression vector comprises at least one RNA polymerase II promoter, for example, a human CMV-immediate early promoter (HCMV-IE) or a simian CMV (SCMV) promoter, at least one RNA polymerase I promoter, or at least one RNA polymerase III promoter. The promoter may also be a T7 promoter, in which case, the cell further comprises T7 RNA polymerase. Alternatively, the promoter may be an SP6 promoter, in which case, the cell further comprises SP6 RNA polymerase. The promoter may also be one convergent T7 promoter and one convergent SP6 RNA promoter. A cell may be made to contain T7 or SP6 polymerase by transforming the cell with a T7 polymerase or an SP6 polymerase expression plasmid, respectively. In some embodiments, a T7 promoter or a RNA polymerase III promoter is operably linked to a nucleic acid that encodes a short dsRNA (e.g., a dsRNA that is less than 200, 150, 100, 75, 50, or 25 basepairs in length). In other embodiments, the promoter is a mitochondrial promoter that allows cytoplasmic transcription of the nucleic acid in the vector (see, for example, the mitochondrial promoters described in WO 00/63364, filed April 19, 2000, and in WO/US2002/00543, filed 9-Jan-2001). Alternatively, the promoter is an inducible promoter, such as a *lac* (Cronin et al. Genes & Development 15: 1506-1517, 2001), *ara* (Khlebnikov et al., J Bacteriol. 2000 Dec;182(24):7029-34), ecdysone (Rheogene website), RU48 (mefepristone) (corticosteroid antagonist) (Wang XJ, Liefer KM, Tsai S, O'Malley BW, Roop DR, Proc Natl Acad Sci U S A. 1999 Jul 20;96(15):8483-8), or *tet* promoter (Rendal et al., Hum Gene Ther. 2002;13(2):335-42 and Larnartina et al., Hum Gene Ther.

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2002;13(2):199-210) or a promoter disclosed in WO 00/63364, filed April 19, 2000. Also useful in the methods and compositions of the invention are the structural and chimeric promoters taught in USSN 60/464,434, filed 22-Apr-2003. See also the promoter systems taught in Pachuk, C., and Satischchandran, C., "Multiple-Compartment Eukaryotic Expression Systems," U.S. Provisional Application No. 60/497,304, filed 22-Aug-2003, which are considered particularly desirable in the methods and compositions of the invention.

Replace the paragraph beginning at page 49, line 17, with the following amended paragraph:

Routes of administration include, but are not limited to, intramuscular, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraocularly and oral as well as transdermally or by inhalation or suppository. Preferred routes of administration include intravenous, intramuscular, oral, intraperitoneal, intradermal, intraarterial and subcutaneous injection.

Add, before the paragraph beginning at page 50, line 3, the following new section:

Targeted transfection of hepatocytes *in vivo* for delivery of dsRNAs against HBV and/or HCV may be accomplished through IV injection with a composition comprising a DNA or RNA expression vector as described herein complexed with a mixture (e.g., a 35%/65% ratio) of a lactosyl spermine (mono or trilactosylated) and cholestryl spermine (containing spermine to DNA at a charge ratio of 0.8). Such compositions are especially useful for pharmaceutical applications and may readily be formulated in a suitable sterile, non-pyrogenic vehicle, e.g., buffered saline for injection, for parenteral administration, e.g., IV (including IV infusion), IM, SC, and for intraperitoneal administration, as well as for aerosolized formulations for pulmonary delivery via inhalation. In certain formulations, a DNA expression construct of the invention may be complexed with an endosomolytic spermine such cholestryl spermine alone, without a targeting spermine; some routes of administration, such as intraperitoneal injection or infusion, may achieve effective hepatic delivery and

transfection of a DNA construct of the invention, and expression of RNA effector molecules, e.g., multiple dsRNA hairpins effective against HBV and/or HCV.

Intraperitoneal administration (e.g., ultrasound guided intraperitoneal injection) of a sterile pharmaceutical composition comprising dsRNA effector molecules and/or dsRNA expression constructs which provide dsRNA effector molecules against HBV and/or HCV in a specially formulated delivery vehicle may be an advantageous route of delivery to promote uptake by liver cells, including hepatocytes. In some compositions the dsRNA expression construct may be complexed with an appropriate transfection enhancing agent, e.g., with a mixture of a lactosyl spermine (mono or trilactosylated) and cholestryl spermine, or in other compositions with an endosomolytic spermine such cholestryl spermine alone, without a targeting spermine. The volume, concentration, and formulation of the pharmaceutical composition as well as the dosage regimen may be tailored specifically to maximize cellular delivery while minimizing toxicity such as an inflammatory response. E.g., relatively large volumes (5, 10, 20, 50 ml or more) with corresponding low concentrations of active ingredients, as well as the inclusion of an anti-inflammatory compound such as a corticosteroid, may be utilized if desired. Formulations as known to those of skill in the art of pharmaceutics may also be utilized to provide sustained release of dsRNA effector molecules and/or dsRNA expression constructs of the invention.

dsRNAs or dsRNA expression constructs may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microprojectile bombardment gene guns". Intraperitoneal injection may be accomplished, e.g., with a traditional syringe, with placement of the needle advantageously guided by ultrasound or a similar technique. Alternatively, the dsRNA and/or dsRNA expression construct may be introduced by various means into cells that are removed from the individual. Such means include, for example, ex vivo transfection, electroporation, microinjection and microprojectile bombardment. After the gene construct is taken up by the cells, they are reimplanted into the individual. It is contemplated that otherwise non-immunogenic cells that have gene constructs incorporated therein can be implanted into the individual even if the host cells were originally taken from another individual.

Replace the paragraph beginning at page 51, line 3, with the following amended paragraph:

For administration in an intact animal, e.g., a human subject infected with HBV and/or HCV, between 1 mg and 100 mg, typically between 1 mg and 10 mg, between 10 ng and 50 µg, between 50 ng and 100 ng, or between 100 ng and 5 µg of dsRNA or DNA encoding one or more dsRNA effector molecules is used. In desirable embodiments, approximately 10 µg of a DNA or 5 µg of dsRNA is administered to the animal. In a desirable embodiment, a pharmaceutical composition for parenteral administration is prepared containing 10 mg of a plasmid dsRNA expression construct of the invention (in some formulations complexed with an appropriate transfection facilitating agent such as cholestryl spermine or a mixture of cholestryl spermine/trilactosyl spermine) in 25 ml of a suitable sterile vehicle for injection such as Normal Saline Injection, D5W, D5%/0.45% NaCl, D5%/0.2% NaCl, etc., and injected intraperitoneally over 5 to 10 minutes, with needle placement guided by ultrasound or a similar technology. Administration may be repeated periodically, e.g., weekly or monthly, as required. With respect to the methods of the invention, it is not intended that the administration of dsRNA or DNA encoding dsRNA to cells or animals be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration sufficient to provide a dose adequate to inhibit gene expression, prevent a disease, or treat a disease.

Add, before the paragraph beginning at page 51, line 18, the following new section:

Therapeutic Advantages of the Invention as it Relates to Worldwide Disease Incidence and Viral Variability

The mutability of the hepatitis C virus genome, and to a lesser but significant extent, the hepatitis B virus genome, has been described above as presenting challenges to the design of nucleic acid based therapeutics against these viral agents. The inventors have painstakingly aligned thousands of individual HCV and HBV sequences, originally deriving from thousands of human viral isolates from widely divergent geographic areas worldwide. In doing so, the instant invention has

identified and specified preferred sequences which are utilized singly and in combination in dsRNA effector molecules which target the least mutable regions of the genome of HCV and/or HBV.

The two-fold rationale for this has been discussed above, primarily in terms of ensuring that during the course of infection of a patient with HBV or HCV, the therapeutic of the invention will remain potent against the virus even as it mutates during the course of disease in a given patient. However, the second part of this rationale for deriving and using highly conserved sequences for design of dsRNA-based therapeutic applications, is that this also increases the certainty that the therapeutic dsRNA effector molecules of the invention, particularly the methods and compositions of the invention which utilize combinations of highly conserved sequences in dsRNA effector molecules against HBV and/or HCV, will be able to treat the viral infection present in individuals from a global variety of different ancestries, genetic makeup, and geographical distribution, which are known to manifest in clusters of viral variants based on such factors. Thus, a key feature of the therapeutic utility and novelty of this invention lies in the method of derivation of the preferred sequences and embodiments, and not simply in the demonstration that any particular HCV or HBV dsRNA sequence can inhibit viral replication of one or a few chosen viral isolates (or their cognate replicons) in a laboratory experiment, i.e., in a cell line or animal model, not necessarily reflective of the broad diversity of the HBV and/or HCV virus worldwide, or even in a particular infected individual over the course of infection.

For example, the hepatitis B virus has four subtypes of surface antigen, namely adw, ayw, adr and ayr. While lamivudine is considered an effective therapy for chronic Hepatitis B, a recent study of HBV resistance demonstrated a 20-fold increase in resistance in the adw subtype, compared to the ayw subtype. B Zollner et al. "20-fold Increase in Risk of Lamivudine [Epivir HBV] Resistance in Hepatitis B Virus Subtype adw"; *The Lancet*. 2001; 357: 934-935. In contrast to such conventional antiviral agents, the dsRNA agents of the invention, (e.g., dsRNA effector molecules and expression constructs of the invention, especially when used in combination as taught herein) which utilize HBV and/or HCV sequences highly conserved across such geographical genetic variants are expected to exhibit highly advantageous antiviral activity.

Similarly, HCV is also known for having a wide range of geographically divergent viral genotypes, subtypes, quasispecies, with the following current general global patterns of genotypes and subtypes:

1a - mostly found in North & South America; also common in Australia
1b - mostly found in Europe and Asia.

2a - is the most common genotype 2 in Japan and China.

2b - is the most common genotype 2 in the US and Northern Europe.

2c - the most common genotype 2 in Western and Southern Europe.

3a - highly prevalent here in Australia (40% of cases) and South Asia.

4a - highly prevalent in Egypt

4c - highly prevalent in Central Africa

5a - highly prevalent only in South Africa

6a - restricted to Hong Kong, Macau and Vietnam

7a and 7b - common in Thailand

8a, 8b & 9a - prevalent in Vietnam

10a & 11a - found in Indonesia

Accordingly, the highly conserved sequences of the invention, which are expected to be conserved among most if not all of these divergent HCV genotypes and subtypes worldwide, including 1a, 1b, 2a, 2b, and 2c, are considered highly effective therapeutics agents, e.g., when utilized as dsRNA effector molecules, especially combinations thereof, and dsRNA expression vectors capable of expressing such dsRNAs.

Replace the section beginning at page 52, line 25, and ending at page 53, line 21, with the following amended section:

Using this model, cells were co-transfected with the infectious molecular clone of HBV and various eiRNA constructs (dsRNA expression constructs). The cells were then monitored for loss of HBV expression and replication as described below. Details on the vector and encoded RNAs used in this experiment are provided at the end of this example.

Experiment 1:

The following is an example of an experiment that was performed using eiRNA vectors (dsRNA expression vectors) encoding sequences derived from GenBank accession number V01460. HBV sequences in these described eiRNA vectors were

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highly conserved sequences identified as described elsewhere herein, which also exhibited activity as siRNAs (See, Pachuk, C., "Methods and Constructs for Evaluation of RNAi targets and Effector Molecules," PCT/US2004/005065, filed 25-Feb-2004). The particular eiRNA backbone vector used for this experiment was a proprietary vector containing a U6 promoter to drive expression of the encoded RNAs. Each vector encoded only one short hairpin RNA (shRNA). The shRNA coding sequence was followed by an RNA pol III termination sequence. Sequences of the U6 promoter, RNA pol III termination signal, and encoded shRNAs are all shown at the end of the example. Similar vectors containing U6 promoters and RNA pol III termination signals are commercially available such as the "siLentGene-2 Cloning Systems" vector from Promega, Inc., Madison, Wis. One of ordinary skill in the art can also create them according to the information provided herein. It is expected that similar results would also be obtained using other expression and promoter systems especially those vectors with RNA pol III promoters that are not U6, for example H1 promoters or 7SK promoters.

Replace Table 1 at page 56 with the following amended Table 1:

Table 1

<u>HBV-AyW coordinates*</u>	<u>SEQ ID NO</u>	<u>Maps within SEQ ID NO</u>
788-808	14	CGTCTGGAGGGGAGTTAGAGAACCTTAACCTCCCTCGCAGACG 5, 6, 7, or 8
807-827	15	TTCTTCCTAGGGACCTGCAGAGAACCTTGAGAAGAAAGAA 5, 6, 7, or 8
1291-1311	16	AAGCCACCCAAAGGACAGCTTAGAGAACCTTAAGCTGTGGCTT 4
1299-1319	17	CAAGGCCACAGC TTGGAGGCTTAGAGAACCTTAAGCCTCCAAGCTGTGCCCTG 4
1737-1757	18	GGATTCAAGGCCGACGGGACGGAGAGAACCTCGTCCCCGTGGCCTGAATCC 10
1907-1927	19	TTCCCGCAAGTATGGATGGCAGAGAGAACCTTGCCGATCCATACTGCGGAA 3
1912-1932	20	CAGTATGGATGGCAGAGGAGAGAACCTCTCCGATCCATACTG 3
1943-1963	21	TCCACGCCATGCCGTGATGCCAGAGAACCTGGCCATCAGCGCATGCGTGGAA 3
1991-2011	22	TGCGTCAGCAAACACTGGCAAGAGAACCTTGCCTGGAAAGTGTGTTGACCGA 3
2791-2811	23	AAAACGCCGGAGACACATCCAAAGAGAACCTTGGATGTTGCTGCCGGTTT 2
2791-2811mut	24	AAAACACCACACGGCATCCAAAGAGAACCTTGGATGCCGGTTT 2
2912-2932	25	TTGAGAGAACGTTCCACCACGGAGAGAACCTCTCGTGGACTTCTCAA 1
2919-2939	26	AAGTCCACCAAGAGTCTAGACAGAGAACCTGGACTCGTGGACTT 1
799-79	49	GCCTCGCAGAGAACGGTCTCAAGAGAACCTTGAGACCTTCGTCGGAGGC

*nucleotide coordinates refer to Genbank accession number V01460.

Add, before the paragraph beginning at page 57, line 5, the following new section:

The HBV sequitopes of Table 1 (without their respective complement sequence and without the "loop" or linker sequence utilized in a "hairpin" or stem-loop dsRNA effector molecule) are shown in Table 1A below. Each such HBV sequitope, together with its complementary sequence, optionally with an appropriate loop or linker sequence as taught herein, may be utilized in a dsRNA effector molecule of the invention (e.g., a duplex dsRNA or hairpin dsRNA effector molecule, or encoded within a dsRNA expression vector). In one aspect, multiple (e.g., 2, 3, 4, 5, 6, or more) of such dsRNA short hairpin effector molecules are encoded in a single expression vector, e.g., a plasmid expression vector, each under the control of a different promoter, e.g., a polymerase III promoter, as described elsewhere herein.

TABLE 1A

<u>HBV-AYW</u> <u>coordinates</u> <small>Genbank accession number V01460*</small>	<u>SEQ</u> <u>ID NO</u>	<u>Conserved HBV Sequitope</u>	<u>Maps within</u> <u>SEQ ID NO</u>
<u>788-808</u>	50	CGTCTGCGAGGCGAGGGAGTT	5, 6, 7, or 8
<u>807-827</u>	51	TTCTTCTTCTAGGGGACCTGC	5, 6, 7, or 8
<u>1291-1311</u>	52	AAGCCACCCAAGGCACAGCTT	4
<u>1299-1319</u>	53	CAAGGCACAGCTTGGAGGCTT	4
<u>1737-1757</u>	54	GGATTCAGCGCCGACGGGACG	10
<u>1907-1927</u>	55	TTCCGCAGTATGGATCGGCAG	3
<u>1912-1932</u>	56	CAGTATGGATCGGCAGAGGAG	3
<u>1943-1963</u>	57	TCCACGCATGCGCTGATGGCC	3
<u>1991-2011</u>	58	TGCGTCAGCAAACACTTGGCA	3
<u>2791-2811</u>	59	AAAACGCCGCAGACACATCCA	2
<u>2912-2932</u>	60	TTGAGAGAAGTCCACCACGAG	1
<u>2919-2939</u>	61	AAGTCCACCAACGAGTCTAGAC	1
<u>799 - 779</u>	62	GCCTCGCAGACGAAGGTCTCA	

Add, before the paragraph beginning at page 66, line 4, the following new section:

A Four-Promoter RNA polymerase III-based expression construct for production of shRNAs which reduce Hepatitis B RNA production and replication.

As described in more detail in PCT/US05/29976 filed 23-August-2005, a plasmid expression vector, pHB4, containing 4 polymerase III promoter short hairpin dsRNA expression cassettes was constructed. Each expression cassette included a polymerase III promoter operably linked to a sequence encoding a short hairpin dsRNA, and a polymerase III termination sequence. The polymerase III promoters were U6, 7SK, and two copies of a 7SK sequence variant promoter, designated 7SK-4A. Each short hairpin dsRNA sequence comprised a double-stranded stem region homologous and complementary to a highly conserved HBV sequence as taught herein. The four dsRNA effector molecules comprised the sequences of SEQ ID NO: 49; SEQ ID NO: 23; SEQ ID NO:19; and SEQ ID NO: 18; which comprise, respectively; the sequences of SEQ ID NO: 62; SEQ ID NO: 59; SEQ ID NO:55; and SEQ ID NO: 54. As described in more detail in Example 1 of PCT/US05/29976, however, the sequence encoding the dsRNA hairpin effector molecule was inserted into an expression cassette of the plasmid expression vector at a restriction site which in effect resulted in several additional nucleotides being added to the 5' end of the ultimate transcript. The predicted transcript which includes the dsRNA hairpin actually contains additional 5' and 3' sequences: a 5' leader consisting of 6 bases (e.g., the Sal I or Hind III or other chosen recognition sequence), followed by the dsRNA hairpin sequences, followed by a short 3' terminal U tract, usually two (1, 2, 3, or 4) U residues incorporated during transcription termination. These differences in length and composition of 5' and 3' transcript sequences flanking the dsRNA hairpin did not appear to adversely affect the ability of the dsRNA hairpin to effect dsRNA-mediated silencing, which suggests that, unlike synthetic dsRNA duplexes, endogenously expressed dsRNA hairpin constructs are effective despite varying in a number of respects, e.g., length of dsRNA "stem" between about 19-29 bp, length and composition of single-stranded loop, presence or absence of additional short 5' and/or 3' sequences.

A luciferase assay as taught in Example 1 of PCT/US05/29976 and in WO 04/076629, published 10-Sept-2004, "Methods and Constructs for Evaluation of RNAi Targets and Effector Molecules" indicated that all 4 promoter/shRNA cassettes were

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active in silencing their target sequences in a cell supplied with the vector and an assayable substrate. The IC₅₀ value decreased substantially when increasing from a one promoter/shRNA cassette vector to the pHB4 expression vector containing 4 promoter/shRNA cassettes. The IC₅₀ values may have also been affected by the relative potency and transcription levels of each shRNA molecule, and did not reflect a simple relationship to the concentration of the vector only, which in effect behaved as four drugs after entering the cell and expressing the four encoded dsRNA molecules. In other words, the increased potency reflected not only the greater number of total shRNA transcripts generated by the vector, but the also the individual potency that each shRNA has to effect the reduction of sAg or eAg production via degradation of the target viral RNA molecules. The progressive addition of shRNA cassettes increased the potency of the vector in an apparently quantitative manner, and furthermore increased the pharmacological activity against the HBV target by inhibiting four distinct sites of the HBV target. It is important to recognize, however, that the ability of the multiple polymerase III expression constructs to express multiple individual antiviral dsRNA hairpin molecules is of significant value in and of itself, not just because of associated increases in "potency". Where the level of antiviral efficacy is high, the incremental quantitative increase in viral inhibition seen with each additional dsRNA molecule may be less important per se than the ability of the constructs to deliver what is in effect a multi-drug regimen, with the inherent advantage of being highly resistant to the development of viral resistance.

The expression vectors, designed to deliver multiple dsRNA effector molecules targeting highly conserved HBV and/or HCV polynucleotide sequences, when delivered to a virally infected cell, have the unique ability to destroy the viral nucleic acid products directly. Moreover, inherent and integral to the design and intent of these multiple promoter vectors (which express a plurality of different inhibitory short hairpin dsRNAs targeting different portions of the viral genome), is the property of generating multiple different viral antagonists simultaneously. The antagonists (short hairpin dsRNA effector molecules) target different genome sequences in the viral genome. One of these antagonists would probably be sufficient to disable the virus; however, the redundancy serves as a "backup" mechanism such that if the viral sequence mutates to render one antagonist inert,

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there are 2, 3, 4 or more additional antagonists available. Additionally, by targeting multiple sites in the viral genome, different DNA or RNA products of the virus which play different roles in the disease pathology can be attacked at the same time.

In the case of Hepatitis B for example, in one embodiment, the instant invention uses 4, 5, or more shRNA molecules selected from the following sequences and other highly conserved HBV sequences as taught herein: e.g., "799" (SEQ ID No. 49); "1907" (SEQ ID No. 19); "2791" (SEQ ID No. 23); "1737" (SEQ ID No. 18), "1991" (SEQ ID No. 22), "1943" (SEQ ID No. 21). Other of the conserved HBV sequences disclosed herein, including sequences of e.g., 19 to 29 nucleotides, which comprise all or part of "799", "1907", "2791", "1737", "1991", or "1943", may be selected for inclusion in dsRNA hairpin effector molecules to be expressed by expression vectors comprising multiple promoters, including multiple polymerase III expression vectors. Due to the nature of HBV gene expression and overlapping transcriptional products this allows targeting of multiple RNA transcripts as well as the replicative template of the virus which will interfere with replication and expression of more than one of the viral proteins. One of the shRNA molecules, "1737" (SEQ ID No. 18) uniquely can disable the RNA encoding a product known as the X protein (HbX). Strong evidence exists in the biomedical literature that the X protein plays a role in establishment and/or maintenance of liver cancer. Because the existing drugs that can to some extent inhibit viral replication cannot eliminate the cell of integrated or other residual copies or portions of the viral genome, these drugs cannot shut off the production of HbX, even in patients "cured" of infectious HBV, and thus can not directly reduce any incidence of cancer that is mediated by dormant HbX. Multiple anti-HBV dsRNA hairpin expression constructs of the present invention can attack both the replication of the virus and the expression of all viral proteins, some which cause the inflammatory insult which results in hepatitis, and others such as HbX, which are believed to promote hepatocellular carcinoma via a distinct but not fully understood mechanism. It is recognized that the principles taught herein can be used to design constructs of the invention specifically tailored to treat such "post infection" patients, which express dsRNAs against Hbx and any other residual HBV antigens.

While the HBV target sequences of the invention were chosen expressly to represent those highly conserved or identical among a large number of different

isolates (strains) of HBV, for reference purposes the identified sequences, e.g., shRNA sequences, can be mapped back to HBV isolate AYW . It should be recognized, therefore, that the dsRNAs and dsRNA expression constructs of the invention are expected to be effective not only against HBV AYW and related viral strains, but against nearly all if not all HBV strains encountered in infected human populations in widely dispersed geographical areas.

Add, before the paragraph beginning at page 69, line 7, the following new section:

Several short duplex dsRNAs comprising the HCV sequences identified above in Table 14 (in each case, the first 21 bases constitute conserved HCV sequences of the invention, followed by an 8-base "adapter" sequence, "CCTGTCTC", appended from the Ambion kit used in synthesis, but which do not appear in the dsRNA effector molecules) targeting the 3'UTR; siRNA #12 targeting the HCV NS5B gene (positive control); the identified HCV core siRNA (positive control); and the identified lamin siRNA (negative control) were synthesized using the Silencer siRNA construction kit, Catalog # 1620 (Ambion Inc., Austin, Tex.). DNA oligonucleotides were synthesized by IDT (Coralville, Iowa).

Table 14A

siRNA number	SEQ ID NO	HCV sequence
#12	63	GCTAAACACTCCAGGCCAATA
#22	64	TCCTTGTTGGCTCCATCTTA
#32	65	GCTCCATCTTAGCCCTAGTCA
#42	66	TCTTAGCCCTAGTCACGGCTA
#52	67	CCTAGTCACGGCTAGCTGTGA
#62	68	CTAGTCACGGCTAGCTGTGAA
#72	69	CGTGAGCCGCTTGACTGCAGA
#82	70	GCTGATACTGGCCTCTCTGCA
#102	71	ACTGGCCTCTCTGCAGATCAA

Replace the paragraph beginning at page 86, line 21, with the following amended paragraph:

This sequence is followed by a loop structure of 9 Ts. The second side of the hairpin contains a sequence that is complementary to the first side of the hairpin. One skilled in the art can easily design and construct hairpin constructs. Note: it is anticipated that other types of eiRNA vectors driven by other promoters, including RNA polymerase III promoters, and encoding other types of RNA structures, including various hairpin structures will have similar effects. Especially desirable are, e.g., "forced" hairpin constructs, partial hairpins capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002 and PCT/US2003/024028, filed 31-Jul-2003, as well as the "udderly" structured hairpins (e.g., multi-hairpin long dsRNA vectors and multi-short hairpin structures), hairpins with mismatched regions, and multiepitope constructs as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, filed 20-Oct-2003, as well as a variety of other dsRNA structures known to those of skill in the art.

Add, before the paragraph beginning at page 87, line 14, the following new section:

EXAMPLE #7

Silencing HBV replication and expression in a replication competent cell culture model (see Example 1) using combinations of HBV- specific eiRNAs in multiple promoter vectors

As disclosed in PCT/US05/29976, filed 23-August-2005 and in U.S. Provisional Applications entitled "Multiple RNA Pol III Promoter Expression Constructs" (Serial No. 60/603622, filed 8/23/04, and Serial No. 60/629942, filed 11/22/04) the teaching of which is hereby incorporated by reference, two or more (preferably 3, 4, 5, 6 or more) of the shRNA sequences shown in Table I and SEQ ID NO:49 may be encoded in the same plasmid vector in separate cistrons under the control of separate promoters for each shRNA. SEQ ID NO:49 is:

GCCTCGCAGACGAAGGTCTCAAGAGAACTTGAGACCTTCGTCTGCGAGGC

SEQ ID NO:49 represents the coding strand of a DNA sequence which encodes an shRNA molecule that targets an HBV conserved region. The first 21 bases of the

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sequence above are identical to the sense sequence of HBV mRNA from position 799 to 779 in the HBV genome, strain AYW (numbered according to the complement strand given in Genbank Accession No. V01460). This sequence is followed by 9 bases (i.e., AGAGAACTT)representing the loop portion of the shRNA, followed by 21 bases of the reverse complementary sequence to the first 21 bases. (It will be understood that the loop sequence serves only to join the complementary sequences which form the double-stranded "stem" and therefore considerable variation in length and nucleotide sequence is acceptable within the loop region.) In a preferred embodiment, this DNA sequence will be placed in an appropriate expression vector operably under the control of a promoter, preferably an RNA polymerase III promoter such 7SK, U6, etc. The resulting RNA transcript:

GCCUCGCAGACGAAGGUCUCAAGAGAACUUUGAGACCUUCGUCUGCGAGGC

will assume a hairpin or stem-loop structure having 21 basepairs in a double-stranded conformation.

Using methods commonly employed by one skilled in the art of molecular biology, a single vector encoding two or more, preferably three or more, more preferably four or more, five or more, or all of SEQ ID NO:49, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, and SEQ ID NO:23 is constructed. A particularly preferred embodiment for pharmaceutical applications of dsRNA-mediated silencing of the HBV target comprises a single expression construct encoding under the control of separate RNA polymerase III promoters, shRNAs corresponding to at least SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:18, and optionally, SEQ ID NO:49 and/or SEQ ID NO:21. Such shRNA-expression vectors may advantageously utilize one or more RNA polymerase III promoters, including U6, 7SK, and H1 promoters in several alternative orientations and combinations. Particularly preferred constructs will utilize one or more of the 7SK promoters as taught in U.S. Provisional Serial Nos. 60/603622 and 60/629942. The instant example is thus analogous to Experiment 1 in Example 1 except that instead of introducing one vector with one shRNA at a time, the applicants deliver a single plasmid construct which expresses multiple shRNAs.

The advantages of this approach for therapeutic applications of dsRNA silencing are principally in the economy, simplicity and coordinated delivery of a single drug entity which comprises multiple different shRNAs each targeting a different site of the HBV genome. The ability to simultaneously target multiple sites of a viral genome is highly advantageous in preventing the clinically widespread phenomenon of drug resistance (by viral mutation), and the ability to combine dsRNA drug entities against these different target sites in a single delivery agent (the plasmid vectors of this invention) makes this conceptual approach uniquely feasible. While shRNAs, e.g., RNAs corresponding to SEQ ID NO:49, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, and SEQ ID NO:23 may be produced, e.g., through chemical synthesis or *in vitro* expression, and delivered into an animal cell singly and in combination, there are significant advantages in some applications to express within the animal cell multiple shRNAs from a single expression vector. In this example, the potency of the multiple shRNA expression vectors significantly exceeded that of any one of the single vectors used in Experiment 1, as measured by similar assays.

EXAMPLE #8

Inhibition of Infectious Virions of HCV by dsRNA Effector Molecules

As a further example of HCV-targeted dsRNAs, the sequences given in Table 16 represent highly conserved coding region sequitopes from the 5' and 3' untranslated regions of HCV. Each sequence is written as the coding strand and is used to specify the design of a short hairpin dsRNA effector molecule comprising the coding sequence as shown in Table 16 connected to its reverse complement by a loop or linker sequence as described elsewhere herein. The sequences shown are predicted to be particularly efficacious as antiviral therapeutic agents because they were tested in a newly available *in vitro* HCV replication system capable of producing whole, infectious virions (disclosed in Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ., "Production of infectious hepatitis C virus in tissue culture from a cloned viral genome", Nat Med 2005 Jul;11 (7):791-6; and in Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV., "Robust hepatitis C virus infection *in vitro*", Proc Natl Acad

Sci USA 2005 Jun 28;102(26):9294-9). In this system, all viral proteins and viral nucleic acid sequences are present in a cell, as in a natural infection. In less complete replicon systems, dsRNA silencing molecules cannot be as rigorously tested as in the new system. It is expected that one or more (2, 3, 4, 5, or more) of the HCV sequitopes and their complements could be utilized as duplex dsRNA effector molecules, short hairpin dsRNA effector molecules, and/or encoded into dsRNA expression vectors capable of expression *in vivo* in a mammalian cell, including a human cell or organism.

Table 16

Seq Name	SEQ ID NO	Sequence (5' to 3')
HCV5M-5.1	72	AAAGGCCTTGTGGTACTGCCT
HCV5M-5.3	73	TTGTGGTACTGCCTGATAGGG
HCVXM-13	74	TAGCTGTGAAAGGTCCGTGAG
HCVXM-34	75	ATCTTAGCCCTAGTCACGGCTAGCTG
HCVXM-35	76	TAGTCACGGCTAGCTGTGAAAGGTCCG

The sequences in Table 17 represent additional preferred highly conserved at least 19 contiguous base pair HCV sequences from the 5' UTR of the virus (SEQ ID NO: 11). To generate the dsRNA effector molecules of the invention, these sequences are used in conjunction with their reverse complement and, optionally, a loop or linker sequence joining the sequence to its reverse complement, when it is desired to form a hairpin dsRNA effector molecule. One or more double-stranded RNA molecules comprising said conserved 5' UTR sequences (from SEQ ID NO: 11) may advantageously be used in combination with one or more other dsRNA effector molecules of the invention, including e.g., one or more highly conserved sequences from the 3' UTR (SEQ ID NO:27) and/or one or more at least 19 contiguous base pair sequences from SEQ ID NO. 12.

Table 17

<u>HCV 5' UTR siRNAs</u>		
<u>Sequence Name</u>	Sequence (5' to 3')	SEQ ID NO
HCV5P-1.1	CCTGTGAGGAACACTACTGTCTT	77
HCV5P-1.2	ACGCAGAAAGCGTCTAGCCAT	78
HCV5P-1.3	CGTCTAGCCATGGCGTTAGTA	79
HCV5P-1.4	GTCTAGCCATGGCGTTAGTAT	80
HCV5P-1.5	CTCCCCTGTGAGGAACACTACTGTCTT	81
HCV5P-1.6	GAGGAACACTACTGTCTTCACGCAGAA	82
HCV5P-1.7	GTGAGGAACACTACTGTCTTCACGCAGAA	83
HCV5P-2.1	GAGCCATAGTGGTCTGCGGAA	84
HCV5P-2.2	GAACCGGTGAGTACACCGGAA	85
HCV5P-2.3	ACCGGTGAGTACACCGGAATT	86
HCV5P-2.4	GGGAGAGCCATAGTGGTCTGCGGAA	87
HCV5P-5.1	GGCCTTGTGGTACTGCCTGAT	88
HCV5P-5.2	GCCTTGTGGTACTGCCTGATA	89
HCV5P-5.3	GTACTGCCTGATAGGGTGCTT	90
HCV5P-5.4	AAGGCCTTGTGGTACTGCCTGATAAGGG	91
HCV5P-5.5	CGAAAGGCCTTGTGGTACTGCCTGATA	92
HCV5P-5.6	CTTGCAGTGCCCCGGGAGGTCTCGTA	93
HCV5M-1.1	ATCACTCCCCTGTGAGGAAC	94
HCV5M-1.2	TTCACGCAGAAAGCGTCTAGC	95
HCV5M-1.3	TAGCCATGGCGTTAGTATGAG	96
HCV5M-1.4	ATCACTCCCCTGTGAGGAACACTG	97
HCV5M-1.5	ATCACTCCCCTGTGAGGAACACTGTC	98
HCV5M-1.6	AACTACTGTCTTCACGCAGAAAGCG	99
HCV5M-1.7	AACTACTGTCTTCACGCAGAAAGCGTC	100
HCV5M-2.1	ATAGTGGTCTGCGGAACCGGT	101
HCV5M-2.2	TAGTGGTCTGCGGAACCGGTG	102
HCV5M-2.3	AACCGGTGAGTACACCGGAATTGCC	103
HCV5M-5.2	AAGGCCTTGTGGTACTGCCTG	104
HCV5M-5.4	TACTGCCTGATAGGGTGCTT	105
HCV5M-5.5	TTGTGGTACTGCCTGATAGGGTGCTT	106
HCV5M-5.6	TACTGCCTGATAGGGTGCTTGCAG	107
HCV5M-5.7	TAGGGTGCTTGCAGTGCCCCGGG	108
HCV5M-5.8	TTGCGAGTGCCCCGGGAGGTCTCGTAG	109

Please replace the Sequence Listing with the attached Sequence Listing.

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

Claims 1-31 (canceled)

Claim 32 (new): A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell at least one double-stranded RNA effector molecule comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T.

Claim 33 (new): The method of claim 32, wherein at least two double-stranded RNA effector molecules are administered to said cell, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO: 49.

Claim 34 (new): The method of claim 33, comprising administering to said cell double-stranded RNA effector molecules comprising SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO: 49.

Claim 35 (new): The method of claim 32, wherein said administering is accomplished by providing to the *in vivo* mammalian cell at least one expression vector capable of expressing at least one double-stranded RNA effector molecule comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:49.

Claim 36 (new): The method of claim 35, comprising providing to the *in vivo* mammalian cell at least one expression vector capable of expressing at least two double-stranded RNA effector molecules comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO: 49.

Claim 37 (new): The method of claim 36, comprising expressing at least four double-stranded RNA effector molecules comprising a sequence selected from the group consisting of SEQ NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:49.

Claim 38 (new): The method of claim 35, wherein said at least one expression vector comprises a promoter selected from the group consisting of an RNA polymerase I promoter, an RNA polymerase II promoter, a T7 polymerase promoter, an SP6 polymerase promoter, an RNA polymerase III promoter, a tRNA promoter, and a mitochondrial promoter, said promoter operably linked to a sequence encoding one or more of said double-stranded RNA effector molecules.

Claim 39 (new): The method of claim 32, wherein the mammalian cell is a human cell.

Claim 40 (new): A composition for inhibiting the expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising at least one double-stranded RNA effector molecule, each double-stranded RNA effector molecule comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T.

Claim 41 (new): A composition of claim 40, comprising at least one expression vector capable of expressing at least one of said double-stranded RNA effector molecules in a mammalian cell.

Claim 42 (new): A composition of claim 41, wherein the expression vector comprises at least one promoter selected from the group consisting of a polymerase I promoter, a polymerase III promoter, a U6 promoter, an H1 promoter, a 7SK promoter, and a mitochondrial promoter, said promoter operably linked to a sequence encoding one or more of said double-stranded RNA effector molecules.

Claim 43 (new): A composition of 40, comprising at least one expression vector capable of expressing in an *in vivo* mammalian cell a double-stranded RNA effector molecule comprising SEQ ID NO:18; a double-stranded RNA effector molecule comprising SEQ ID NO:19; a double-stranded RNA effector molecule comprising SEQ ID NO:23; and a double-stranded RNA effector molecule comprising SEQ ID NO:49; wherein U is substituted for T.

Claim 44 (new): A mammalian cell comprising an expression vector of claim 41.

Claim 45 (new): A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell at least two double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T.

Claim 46 (new): The method of claim 45, wherein said at least two double-stranded RNA effector molecules are administered to the cell by providing at least one expression vector encoding the double-stranded RNA effector molecules.

Claim 47 (new): The method of claim 46, wherein the double-stranded RNA effector molecules are hairpin dsRNA molecules.

Claim 48 (new): The method of claim 46, wherein the expression vector comprises at least one promoter selected from the group consisting of a polymerase I promoter, a polymerase III promoter, a U6 promoter, an H1 promoter, a 7SK promoter, and a mitochondrial promoter, said promoter operably linked to a sequence encoding one or more of said double-stranded RNA effector molecules.

Claim 49 (new): A composition for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising at least two double-stranded RNA effector molecules, each double-stranded RNA effector

molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T.

Claim 50 (new): The composition of claim 49, comprising at least one expression vector encoding said at least two double-stranded RNA effector molecules.

Claim 51 (new): The composition of claim 50, wherein the double-stranded RNA effector molecules are hairpin dsRNA molecules.

Claim 52 (new): The composition of claim 50, wherein the expression vector comprises at least one promoter selected from the group consisting of a polymerase I promoter, a polymerase III promoter, a U6 promoter, an H1 promoter, a 7SK promoter, and a mitochondrial promoter, said promoter operably linked to a sequence encoding one or more of said double-stranded RNA effector molecules.

Claim 53 (new): A method for inhibiting expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering to said cell at least two double-stranded RNA effector molecules comprising: (a) an RNA sequence equivalent to a hepatitis C virus DNA coding strand sequence selected from the group consisting of sequence position 9510-9531, 9510-9533, 9510-9534, 9510-9535, 9510-9536, 9514-9534, 9514-9535, 9514-9536, 9514-9539, 9514-9540, 9514-9542, 9517-9539, 9517-9540, 9517-9542, 9517-9544, 9518-9539, 9518-9540, 9518-9542, 9518-9544, 9520-9540, 9520-9542, 9520-9544, 9520-9548, 9521-9542, 9521-9544, 9521-9548, 9521-9549, 9522-9542, 9522-9544, 9522-9548, 9522-9549, 9527-9548, 9527-9549, 9527-9551, 9527-9552, 9527-9553, 9527-9555, 9528-9548, 9528-9549, 9528-9551, 9528-9552, 9528-9553, 9528-9555, 9530-9551, 9530-9552, 9530-9553, 9530-9555, 9530-9557, 9530-9558, 9532-9552, 9532-9553, 9532-9555, 9532-9557, 9532-9558, 9532-9559, 9532-9560, 9537-9557, 9537-9558, 9537-9559, 9537-9560, 9537-9561, 9537-9564, 9538-9558, 9538-9559, 9538-9560, 9538-9561, 9538-9564, 9538-9566, 9541-9561, 9541-9564, 9541-9566, 9541-9568, 9541-9569, 9543-9564, 9543-9566, 9543-9568, 9543-9569, 9543-9571, 9545-9566, 9545-9568,

9545-9569, 9545-9571, 9545-9573, 9546-9564, 9546-9566, 9546-9569, 9546-9571, 9546-9573, 9547-9568, 9547-9569, 9547-9571, 9547-9573, 9547-9575, 9550-9571, 9550-9573, 9550-9575, 9550-9577, 9550-9578, 9554-9575, 9554-9577, 9554-9578, 9554-9580, 9556-9577, 9556-9578, 9556-9580, 9556-9584, 9562-9584, 9562-9586, 9562-9587, 9562-9588, 9562-9589, 9563-9584, 9563-9586, 9563-9587, 9563-9588, 9563-9589, 9563-9591, 9565-9586, 9565-9587, 9565-9588, 9565-9589, 9565-9591, 9565-9593, 9567-9587, 9567-9588, 9567-9589, 9567-9591, 9567-9593, 9567-9595, 9570-9591, 9570-9593, 9570-9595, 9570-9596, 9570-9598, 9572-9593, 9572-9595, 9572-9596, 9572-9598, 9574-9595, 9574-9596, 9574-9598, 9574-9601, 9576-9596, 9576-9601, 9576-9604, 9579-9601, 9579-9604, 9581-9601, 9581-9604, and 9583-9604; (b) an RNA sequence which is the reverse complement of the selected hepatitis C virus DNA coding strand sequence; and, optionally, (c) a sequence linking (a) and (b).

Claim 54 (new): The method of claim 53, wherein said administering is accomplished by providing one or more expression vectors capable of expressing in said mammalian cell said at least two double-stranded RNA effector molecules.

Claim 55 (new): The method of claim 54, wherein said one or more expression vectors comprise at least one promoter selected from an RNA polymerase I promoter, an RNA polymerase II promoter, a T7 polymerase promoter, an SP6 polymerase promoter, an RNA polymerase III promoter, a tRNA promoter, and a mitochondrial promoter, said promoter operably linked to a sequence encoding at least one of said double-stranded RNA effector molecules.

Claim 56 (new): The method of Claim 55, wherein at least one expression vector comprises at least two expression cassettes, each expression cassette comprising at least one RNA polymerase III promoter selected from the group consisting of a U6 promoter, a 7SK promoter, an H1 promoter, and an MRP promoter.

Claim 57 (new): The method of claim 53, wherein the mammalian cell is a human cell.

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Claim 58 (new): A composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising at least two double-stranded RNA effector molecules, each comprising: (a) an RNA sequence equivalent to a hepatitis C virus DNA coding strand sequence selected from the group consisting of sequence position 9510-9531, 9510-9533, 9510-9534, 9510-9535, 9510-9536, 9514-9534, 9514-9535, 9514-9536, 9514-9539, 9514-9540, 9514-9542, 9517-9539, 9517-9540, 9517-9542, 9517-9544, 9518-9539, 9518-9540, 9518-9542, 9518-9544, 9520-9540, 9520-9542, 9520-9544, 9520-9548, 9521-9542, 9521-9544, 9521-9548, 9521-9549, 9522-9542, 9522-9544, 9522-9548, 9522-9549, 9527-9548, 9527-9549, 9527-9551, 9527-9552, 9527-9553, 9527-9555, 9528-9548, 9528-9549, 9528-9551, 9528-9552, 9528-9553, 9528-9555, 9530-9551, 9530-9552, 9530-9553, 9530-9555, 9530-9557, 9530-9558, 9532-9552, 9532-9553, 9532-9555, 9532-9557, 9532-9558, 9532-9559, 9532-9560, 9537-9557, 9537-9558, 9537-9559, 9537-9560, 9537-9561, 9537-9564, 9538-9558, 9538-9559, 9538-9560, 9538-9561, 9538-9564, 9538-9566, 9541-9561, 9541-9564, 9541-9566, 9541-9568, 9541-9569, 9543-9564, 9543-9566, 9543-9568, 9543-9569, 9543-9571, 9545-9566, 9545-9568, 9545-9569, 9545-9571, 9545-9573, 9546-9564, 9546-9566, 9546-9569, 9546-9571, 9546-9573, 9547-9568, 9547-9569, 9547-9571, 9547-9573, 9547-9575, 9550-9571, 9550-9573, 9550-9575, 9550-9577, 9550-9578, 9554-9575, 9554-9577, 9554-9578, 9554-9580, 9556-9577, 9556-9578, 9556-9580, 9556-9584, 9562-9584, 9562-9586, 9562-9587, 9562-9588, 9562-9589, 9563-9584, 9563-9586, 9563-9587, 9563-9588, 9563-9589, 9563-9591, 9565-9586, 9565-9587, 9565-9588, 9565-9589, 9565-9591, 9565-9593, 9567-9587, 9567-9588, 9567-9589, 9567-9591, 9567-9593, 9567-9595, 9570-9591, 9570-9593, 9570-9595, 9570-9596, 9570-9598, 9572-9593, 9572-9595, 9572-9596, 9572-9598, 9574-9595, 9574-9596, 9574-9598, 9574-9601, 9576-9596, 9576-9598, 9576-9601, 9576-9604, 9579-9601, 9579-9604, 9581-9601, 9581-9604, and 9583-9604; (b) an RNA sequence which is the reverse complement of the selected hepatitis C virus DNA coding strand sequence; and, optionally, (c) a sequence linking (a) and (b).

Claim 59 (new): An expression vector encoding a composition of claim 58.

Claim 60 (new): A mammalian cell comprising an expression construct of claim 59.

Claim 61 (new): An expression vector of claim 59 comprising at least one promoter selected from an RNA polymerase I promoter, an RNA polymerase II promoter, a T7 polymerase promoter, an SP6 polymerase promoter, an RNA polymerase III promoter, a tRNA promoter, and a mitochondrial promoter, said promoter operably linked to a sequence encoding at least one of said double-stranded RNA effector molecules.

Claim 62 (new): An expression vector of claim 61 comprising at least two expression cassettes, each expression cassette comprising at least one RNA polymerase III promoter selected from the group consisting of a U6 promoter, a 7SK promoter, an H1 promoter, and a MRP promoter, each of said RNA polymerase III promoters operably linked to a sequence encoding a double-stranded RNA effector molecule.

Claim 63 (new): A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T.

Claim 64 (new): The method of claim 63, wherein at least two of said double-stranded RNA effector molecules are administered to the same mammalian cell.

Claim 65 (new): The method of claim 64, wherein said at least two double-stranded RNA effector molecules comprise an at least 19 contiguous base pair nucleotide sequence from within more than one of SEQ ID NO:1 through SEQ ID NO:10.

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Claim 66 (new): The method of claim 65, wherein said administering is accomplished by providing one or more expression vectors capable of expressing in said mammalian cell said at least two double-stranded RNA effector molecules.

Claim 67 (new): The method of claim 66, wherein said one or more expression vectors further comprise a promoter selected from an RNA polymerase I promoter, an RNA polymerase II promoter, a T7 polymerase promoter, an SP6 polymerase promoter, an RNA polymerase III promoter, a tRNA promoter, and a mitochondrial promoter, said promoter operably linked to a sequence encoding at least one of said double-stranded RNA effector molecules.

Claim 68 (new): A method for inhibiting expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering to said cell a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T.

Claim 69 (new): The method of claim 68, wherein at least two of said double-stranded RNA effector molecules are administered to the same mammalian cell.

Claim 70 (new): The method of claim 69, wherein said at least two double-stranded effector molecules comprise an at least 19 contiguous base pair nucleotide sequence from within more than one of SEQ ID NO:11; SEQ ID NO:12; and SEQ ID NO: 27.

Claim 71 (new): The method of claim 70, wherein said administering is accomplished by providing one or more expression vectors capable of expressing in said mammalian cell said at least two double-stranded RNA effector molecules.

Claim 72 (new): The method of claim 71, wherein said one or more expression vectors comprise one or more promoters selected from an RNA polymerase I promoter, an RNA polymerase II promoter, a T7 polymerase promoter, an SP6 polymerase promoter, an RNA polymerase III promoter, a tRNA promoter, and a

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mitochondrial promoter, said promoter operably linked to a sequence encoding at least one of said double-stranded RNA effector molecules.

Claim 73 (new): The method of claim 72, wherein at least one expression vector comprises at least two expression cassettes, each expression cassette comprising at least one RNA polymerase III promoter selected from the group consisting of a U6 promoter, a 7SK promoter, an H1 promoter, and a MRP promoter, each of said RNA polymerase III promoters operably linked to a sequence encoding a said double-stranded RNA effector molecule.

Claim 74 (new): A method for inhibiting expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in the same *in vivo* mammalian cell, comprising administering to said cell a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11; SEQ ID NO:12; and SEQ ID NO: 27; wherein U is substituted for T.

Claim 75 (new): The method of claim 74, wherein at least two double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; and at least two double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO: 27, are administered to the same *in vivo* mammalian cell.

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Claim 76 (new): The method of claim 74, wherein said administering is accomplished by providing one or more expression vectors capable expressing said double-stranded RNA effector molecules in said mammalian cell.

Claim 77 (new): The method of claim 76, wherein said one or more expression vectors comprise one or more promoters selected from the group consisting of an RNA polymerase I promoter, an RNA polymerase II promoter, a T7 polymerase promoter, an SP6 polymerase promoter, an RNA polymerase III promoter, a tRNA promoter, and a mitochondrial promoter, said promoter(s) operably linked to a sequence encoding at least one of said double-stranded RNA effector molecules.

Claim 78 (new): A composition for inhibiting the expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T.

Claim 79 (new): The composition of claim 78 comprising at least two double-stranded RNA effector molecules wherein said effector molecules comprise an at least 19 contiguous base pair nucleotide sequence from within more than one of SEQ ID NO:1 through SEQ ID NO:10.

Claim 80 (new): A composition of claim 79, comprising at least two double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within more than one of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8.

Claim 81 (new): A composition of claim 80, comprising at least three double-stranded RNA effector molecules each comprising an at least 19 contiguous base pair nucleotide sequence from within at least three of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8.

Claim 82 (new): A composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T.

Claim 83 (new): The composition of claim 82 comprising at least two double-stranded RNA effector molecules, wherein effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within more than one of SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO: 27 are present in the composition.

Claim 84 (new): The composition of claim 83 comprising at least one expression construct capable of expressing the at least two double stranded RNA effector molecules in an *in vivo* mammalian cell.

Claim 85 (new): A composition for inhibiting the expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in a single *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:27; wherein U is substituted for T.

Claim 86 (new): The composition of claim 85 comprising at least one expression construct capable of expressing the at least two double stranded RNA effector molecules in an *in vivo* mammalian cell.

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Claim 87 (new): The composition of claim 85 comprising at least two double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; and at least two double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO: 27.

Claim 88 (new): The composition of claim 87 comprising at least one expression vector capable of expressing said double-stranded RNA effector molecules.

Claim 89 (new): The composition of claim 87 comprising at least two double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8.

Claim 90 (new): A composition of claim 89 comprising at least one expression vector capable of expressing said at least two double-stranded RNA effector molecules in an *in vivo* mammalian cell.

Claim 91 (new): A polynucleotide sequence comprising a sequence selected from SEQ ID NO:14 through SEQ ID NO:26, and SEQ ID NO:49.

Claim 92 (new): A polynucleotide sequence comprising nucleotides 1-19, 1-20, 1-21, 2-20, 2-21, or 3-21 of a sequence selected from SEQ ID NO:14 through SEQ ID NO:26, and SEQ ID NO:49.

Claim 93 (new): A polynucleotide sequence comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from SEQ ID NO:27 through SEQ ID NO:44, SEQ ID NO: 50 through SEQ ID NO:62, and SEQ ID NO: 72 through 76.

Claim 94 (new): A composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in a mammalian cell, comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:27; wherein U is substituted for T.

Claim 95 (new): A composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in a mammalian cell, comprising at least one double-stranded RNA effector molecule comprising (a) a sequence selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, and SEQ ID NO: 76, and (b) the reverse complement of said selected sequence; and, optionally, (c) a sequence linking sequences (a) and (b); wherein U is substituted for T.

Claim 97 (new): A composition of claim 95 wherein said at least one double-stranded RNA effector molecule comprises a sequence selected from the group consisting of SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, and SEQ ID NO: 76.

Claim 98 (new): An expression vector encoding a double-stranded RNA effector molecule of claim 95.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: PACHUK et al. Confirmation No.: Not yet assigned

Application No.: CIP of PCT/2004/019229 Group No.: Not yet assigned
(Generated by petition
under 37 CFR 1.182,
filed concurrently
herewith)

Filed: 06/19/2006 Examiner: Not yet assigned

For: CONSERVED HBV AND HCV SEQUENCES USEFUL FOR GENE
SILENCING

MAIL STOP PETITIONS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PRELIMINARY AMENDMENT

Dear Sir:

This is filed in conjunction with a petition under 37 C.F.R. 1.182 to generate a separate Continuation-In-Part application from the parent PCT application PCT/2004/019229 properly claiming the benefit of priority of US Provisional Application No. 60/638,294 filed December 22, 2004 and U.S. Provisional Application No. 60/478,076, filed June 12, 2003 from the application currently pending as U.S. 10/560,377.

Amendments to the claims are shown in the **Listing of the Claims** begins on **page 2** of this paper.

Amendments to the **Figures** are included in new Figure 15 enclosed herewith.

Remarks begin on **page 5** of this paper.

Listing of the Claims:

The following listing of the claims is to replace all previous listings of the claims. Claims are numbered relative to claims in the parent PCT/US2004/019229 application, from which this application is being generated.

Claims 1-31. (CANCELED)

Claim 32. (NEW) A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell at least two double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T.

Claim 33. (NEW) The method of claim 32, wherein said at least two double-stranded RNA effector molecules are administered to the cell by providing at least one expression vector encoding the double-stranded RNA effector molecules.

Claim 34. (NEW) The method of claim 32, wherein the double-stranded RNA effector molecules are hairpin dsRNA molecules.

Claim 35. (NEW) The method of claim 33, wherein the expression vector comprises at least one promoter selected from the group consisting of a polymerase I promoter, a polymerase III promoter, a U6 promoter, an H1 promoter, a 7SK promoter, and a mitochondrial promoter, said promoter operably linked to a sequence encoding one or more of said double-stranded RNA effector molecules.

Claim 36. (NEW) A composition for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising at least two double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T.

Claim 37. (NEW) The composition of claim 36, comprising at least one expression vector encoding said at least two double-stranded RNA effector molecules.

Claim 38. (NEW) The composition of claim 36, wherein the double-stranded RNA effector molecules are hairpin dsRNA molecules.

Claim 39. (NEW) A method for inhibiting expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in the same *in vivo* mammalian cell, comprising administering to said cell a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11; SEQ ID NO:12; and SEQ ID NO: 27; wherein U is substituted for T.

Claim 40. (NEW) The method of claim 39, wherein at least two double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:1 , SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; and at least two double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO: 27, are administered to the same *in vivo* mammalian cell.

Claim 41. (NEW) The method of claim 39, wherein said administering is accomplished by providing one or more expression vectors capable expressing said double-stranded RNA effector molecules in said mammalian cell.

Claim 42. (NEW) The method of claim 41, wherein said one or more expression vectors comprise one or more promoters selected from the group consisting of an RNA polymerase I promoter, an RNA polymerase II promoter, a T7 polymerase promoter, an SP6 polymerase promoter, an RNA polymerase III promoter, a tRNA promoter, and a mitochondrial promoter, said promoter(s) operably linked to a sequence encoding at least one of said double-stranded

RNA effector molecules.

Claim 43. (NEW) A composition for inhibiting the expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in a single *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:27; wherein U is substituted for T.

Claim 44. (NEW) The composition of claim 43 comprising at least one expression vector capable of expressing the at least two double stranded RNA effector molecules in an *in vivo* mammalian cell.

Claim 45. (NEW) The composition of claim 43 comprising at least two double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:1 , SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ 1D NO:8, SEQ ID NO:9, and SEQ ID NO:10; and at least two double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO: 27.

Claim 46. (NEW) The composition of claim 45 comprising at least one expression vector capable of expressing said double-stranded RNA effector molecules.

REMARKS

Claims 1-31 originally pending in the parent PCT are cancelled and new claims 32-46 are added herein. The new claims are fully supported in the specification. Entry and consideration of the new claims is respectfully requested.

New Figure 15 is enclosed herewith. Figure 15 is drawn from U.S. provisional priority application 60/638,294, which was incorporated by reference in the original U.S. Utility filing of December 12, 2005.

Should any fees be associated with this submission, Applicants authorize the Commissioner to charge such fees to Nixon Peabody Deposit Account No. 50-0850, making reference to Docket No. 051058-034000. Any overpayments should also be credited to said Deposit Account.

Respectfully submitted,

Date: September 9, 2010

/Mark J. FitzGerald/

Mark J. FitzGerald (Reg. No. 45,928)
Attorney for Applicant
NIXON PEABODY LLP
100 Summer Street
Boston, MA 02110
(617) 345-1058

TITLE
CONSERVED HBV AND HCV SEQUENCES USEFUL
FOR GENE SILENCING

CROSS-REFERENCE TO RELATED APPLICATION

5 ~~This application claims the benefit of U.S. Provisional Application Serial No. 60/478,076 filed on June 12, 2003, which is hereby incorporated by reference in its entirety.~~

10 ~~This application is a Continuation-in-part under 35 USC §120 of International Application PCT/US2004/019229, filed June 10, 1994, which claims the benefit of priority of U.S. Provisional Application 60/478,076, filed June 12, 2003, the entireties of both of which are incorporated herein by reference in their entireties. This application also claims the benefit under 35 USC §119(e) of US Provisional Application No. 60/638,294, filed December 22, 2004, which is incorporated herein by reference in its entirety.~~

FIELD OF THE INVENTION

15 This invention relates to methods and compositions utilizing conserved genetic sequences of known hepatitis B viral (HBV) strains and known hepatitis C viral (HCV) strains to modulate the expression of HBV and/or HCV in mammalian cells, via double-stranded RNA-mediated gene silencing, including post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS).

BACKGROUND OF THE INVENTION

20 Human hepatitis C (HCV) is a major public health problem with an estimated 200 million persons worldwide infected. The number of new infections per year in the United States is estimated to be about 25,000 in 2001. This number has declined from an estimated 240,000 new cases per year in the 1980's due to blood donor screening. Nevertheless, an estimated 3.9 million (1.8%) Americans have been infected with HCV, of whom 2.7 million are chronically infected.. Hepatitis C shows significant genetic variation in worldwide populations, evidence of its frequent rates of mutation and rapid evolution. There are six basic genotypes of HCV, with 15 recorded subtypes, which vary in prevalence across different regions of the world. Each of these major genotypes may differ significantly in their biological effects - in terms of replication,

mutation rates, type and severity of liver damage, and detection and treatment options - however, these differences are not yet clearly understood.

There is currently no vaccine against HCV and available drug therapy, including ribavirin and interferon, is only partially effective. It is estimated that 75-85% of infected persons will 5 develop a chronic infection, with 70% of chronically infected persons expected to develop chronic liver 5 disease including hepatocellular carcinoma. Chronic HCV related liver disease is a leading indication for liver transplant.

Although a human hepatitis B vaccine has been available since 1982, it is estimated that 350 million persons worldwide are chronically infected with HBV. While the number of new 10 infections per year in the United States has declined from an average of 260,000 in the 1980s to about 78,000 in 2001, there are an estimated 1.25 million hepatitis B carriers, defined as persons positive for hepatitis B surface antigen (HBsAg) for more than 6 months. Such carriers of HBV are at increased risk for developing cirrhosis, hepatic decompensation, and hepatocellular carcinoma. Although most carriers do not develop hepatic complications from chronic hepatitis 15 B, 15% to 40% will develop serious sequelae during their lifetime, and death from chronic liver disease occurs in 15-25% of chronically infected persons.

There is a need for improved therapeutic agents effective in patients suffering from HBV and/or HCV infection, especially chronic infection, which together are estimated to account for 75% of all cases of liver disease around the world. There is also an extreme need for 20 prophylactic methods and agents effective against HCV.

Nucleic acids (e.g., DNA, RNA, hybrid, heteroduplex, and modified nucleic acids) have come to be recognized as extremely valuable agents with significant and varied biological activities, including their use as therapeutic moieties in the prevention and/or treatment of disease states in man and animals. For example, oligonucleotides acting through antisense 25 mechanisms are designed to hybridize to target mRNAs, thereby modulating the activity of the mRNA. Another approach to the utilization of nucleic acids as therapeutics is designed to take advantage of triplex or triple strand formation, in which a single-stranded oligomer (e.g., DNA or RNA) is designed to bind to a double-stranded DNA target to produce a desired result, e.g., inhibition of transcription from the DNA target. Yet another approach to the utilization of 30 nucleic acids as therapeutics is designed to take advantage of ribozymes, in which a structured RNA or a modified oligomer is designed to bind to an RNA or a double-stranded DNA target to

produce a desired result, e.g., targeted cleavage of RNA or the DNA target and thus inhibiting its expression. Nucleic acids may also be used as immunizing agents, e.g., by introducing DNA molecules into the tissues or cells of an organism that express proteins capable of eliciting an immune response. Nucleic acids may also be engineered to encode an RNA with antisense, 5 ribozyme, or triplex activities, or to produce RNA that is translated to produce protein(s) that have biological function.

More recently, the phenomenon of RNAi or double-stranded RNA (dsRNA)-mediated gene silencing has been recognized, whereby dsRNA complementary to a region of a target gene in a cell or organism inhibits expression of the target gene (see, e.g., WO 99/32619, published 10 July 1999, Fire et al.; and U.S. 6,506,559: "Genetic Inhibition by Double-Stranded RNA;" WO 00/63364: "Methods and Compositions for Inhibiting the Function of Polynucleotide Sequences," Pachuk and Satishchandran; and U.S.S.N. 60/419,532, filed October 18, 2002). dsRNA-mediated gene silencing, utilizing compositions providing an at least partially double-stranded dsRNA, is expected to provide extremely valuable therapeutic and/or prophylactic 15 agents against viral infection, including HBV and/or HCV, including in the extremely difficult problem of chronic HBV and/or HCV infection.

SUMMARY OF THE INVENTION

Applicants' invention provides a method for inhibiting expression of 30 a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell 20 a double stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. In a preferred embodiment of the method, effector sequences from more than one SEQ ID sequence may be 25 administered to the same cell, and/or more than one effector sequence from within the same SEQ ID sequence may be administered to the same cell.

Applicants' method further provides a method for inhibiting expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering to said cell a double stranded RNA effector molecule comprising comprising an at 30 least 19 contiguous base pair nucleotide sequence from within a sequence selected from the

group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T. In a preferred embodiment of this aspect of the method, effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 may be administered to the same cell; and/or more than one effector molecule from within the same SEQ ID NO may be administered to the same cell.

5 Applicants' method further provides A method for inhibiting expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in the same *in vivo* mammalian cell, comprising administering to said cell a double stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ
10 ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T. In preferred embodiments of this aspect of the invention, effector molecules
15 from more than one of SEQ ID NO:1 through SEQ ID NO:10 may be administered to the same cell; and/or effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 may be administered to the same cell; and/or more than one effector molecules from within the same SEQ ID NO may be administered to the same cell.

Applicants' invention further provides a composition for inhibiting the expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising a double stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. Preferred
20 embodiments of the composition include wherein effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO is present in the composition.
25

Applicants' invention further provides a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising a double stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ
30 ID NO:12; wherein U is substituted for T. In a preferred embodiment of this aspect of the invention, effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 may be administered to the same cell; and/or more than one effector molecule from within the same SEQ ID NO may be administered to the same cell.

~~ID NO:12; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO may be present in the composition.~~

5 Applicants' invention further provides a composition for inhibiting the expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in a single *in vivo* mammalian cell comprising a double stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID
10 NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from more than
15 one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 are present in the composition; and/or wherein more than one effector sequence from within the same SEQ ID NO may be present in the composition.

20 In particularly preferred embodiments of the above methods and compositions of the invention, the polynucleotide sequence is RNA, and the mammalian cell is a human cell.

25 Further provided are compositions for inhibiting the expression of a polynucleotide sequence of hepatitis B virus and/or a polynucleotide sequence of hepatitis C virus in mammalian cells, wherein said compositions comprise an at least 19 contiguous nucleotide sequence selected from within SEQ ID NO:1 through SEQ ID NO:12, the complement sequences of SEQ ID NO:1 through SEQ ID NO:12, and mixtures of these sequences. In this embodiment of the invention, the "an at least 19 contiguous nucleotide sequence" is preferably DNA, and the mammalian cell is preferably human. Also provided are expression constructs comprising any of the aforementioned compositions and a mammalian cell comprising said expression constructs.

30 A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell at least one double-stranded

RNA effector molecule, preferably 2, 3, 4, 5, 6, or more double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T. In a preferred method, three or
5 four dsRNA effector molecules, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T; are administered to an *in vivo* mammalian cell. The double-stranded RNA effector molecules may be prepared exogenously and administered into a mammalian cell or expressed intracellularly in a mammalian cell from a double-stranded RNA expression vector,
10 i.e., an expression vector engineered to express a dsRNA effector molecule in a mammalian cell. In a preferred method, at least three or four dsRNA effector molecules, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T; are encoded in a dsRNA expression vector which is administered to an *in vivo* mammalian cell.
15 A composition for inhibiting the expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising at least one, preferably 2, 3, 4, 5, 6 or more double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted
20 for T. In a preferred composition, at least three or four dsRNA effector molecules are included, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T. The double-stranded RNA effector molecules may be prepared exogenously and the composition comprising two, three, four, five, six, or more dsRNA effector molecules administered into a
25 mammalian cell, or the composition may comprise one or more dsRNA expression constructs capable of expressing in a mammalian cell two, three, four, five, six or more of said dsRNA effector molecules. In a preferred composition, three or four dsRNA effector molecules, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T, are encoded in a dsRNA
30 expression vector.

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell at least two, preferably 3, 4, 5, 6 or more, double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 54, SEQ ID NO:

5 55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In a preferred method, said dsRNA effector molecules will comprise 3 or 4 sequences selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:59, and SEQ ID NO:62; wherein U is substituted for T. The double-stranded

10 RNA effector molecules may be stem-loop or hairpin structures and/or duplex double-stranded RNA molecules. The double-stranded RNA effector molecules may be prepared exogenenously and the two, three, four, five, six, or more dsRNA effector molecules administered into a mammalian cell, or one or more dsRNA expression constructs capable of expressing in a mammalian cell two, three, four, five, six or more of said dsRNA effector

15 molecules may be administered.

A composition for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising at least two double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID

20 NO:59, and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In a preferred composition, three or four of said dsRNA effector molecules will be included, or encoded in an expression vector, comprising sequences selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:59, and SEQ ID NO:62; wherein U is substituted

25 for T. The double-stranded RNA effector molecules may be prepared exogenously and the composition will comprise two, three, four, five, six, or more of said dsRNA effector molecules for administration into a *in vivo* mammalian cell, or the composition may comprise one or more dsRNA expression constructs capable of expressing in a mammalian cell two, three, four, five, six or more of said dsRNA effector molecules.

30 In another aspect the invention relates to methods and compositions for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell

comprising administering to said cell at least two, preferably 3, 4, 5, 6 or more, double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 50; SEQ ID NO: 51; SEQ ID NO: 52; SEQ ID NO:53; SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56; SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO: 60; SEQ ID NO:61; and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T.

A polynucleotide sequence comprising SEQ ID NO:49.

A method for inhibiting expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering to said cell at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6, or more double-stranded RNA effector molecules, comprising (a) an RNA sequence equivalent to a hepatitis C virus DNA coding strand sequence selected from the group consisting of sequence position 9510-9531, 9510-9533, 9510-9534, 9510-9535, 9510-9536, 9514-9534, 9514-9535, 9514-9536, 9514-9539, 9514-9540, 9514-9542, 9517-9539, 9517-9540, 9517-9542, 9517-9544, 9518-9539, 9518-9540, 9518-9542, 9518-9544, 9520-9540, 9520-9542, 9520-9544, 9520-9548, 9521-9542, 9521-9544, 9521-9548, 9521-9549, 9522-9542, 9522-9544, 9522-9548, 9522-9549, 9527-9548, 9527-9549, 9527-9551, 9527-9552, 9527-9553, 9527-9555, 9528-9548, 9528-9549, 9528-9551, 9528-9552, 9528-9553, 9528-9555, 9530-9551, 9530-9552, 9530-9553, 9530-9555, 9530-9557, 9530-9558, 9532-9552, 9532-9553, 9532-9555, 9532-9557, 9532-9558, 9532-9559, 9532-9560, 9537-9557, 9537-9558, 9537-9559, 9537-9560, 9537-9561, 9537-9564, 9538-9558, 9538-9559, 9538-9560, 9538-9561, 9538-9564, 9538-9566, 9541-9561, 9541-9564, 9541-9566, 9541-9568, 9541-9569, 9543-9564, 9543-9566, 9543-9568, 9543-9569, 9543-9571, 9545-9566, 9545-9568, 9545-9569, 9545-9571, 9545-9573, 9546-9564, 9546-9566, 9546-9569, 9546-9571, 9546-9573, 9547-9568, 9547-9569, 9547-9571, 9547-9573, 9547-9575, 9550-9571, 9550-9573, 9550-9575, 9550-9577, 9550-9578, 9554-9577, 9554-9578, 9554-9580, 9556-9577, 9556-9578, 9556-9580, 9556-9584, 9562-9584, 9562-9586, 9562-9587, 9562-9588, 9562-9589, 9563-9584, 9563-9586, 9563-9587, 9563-9588, 9563-9589, 9563-9591, 9565-9586, 9565-9587, 9565-9588, 9565-9589, 9565-9591, 9565-9593, 9567-9587, 9567-9588, 9567-9589, 9567-9591, 9567-9593, 9567-9595, 9570-9591, 9570-9593, 9570-9595, 9570-9596, 9570-9598, 9572-9593, 9572-9595, 9572-9596, 9572-9598, 9574-9595, 9574-9596, 9574-9598, 9574-9601, 9576-9596, 9576-9598, 9576-9601, 9576-9604, 9579-

9601, 9579-9604, 9581-9601, 9581-9604, and 9583-9604 and (b) an RNA sequence which is the reverse complement of the selected sequence equivalent to the hepatitis C virus DNA coding strand sequence. In some embodiments, said RNA sequences (a) and (b) are linked by a loop sequence and the double-stranded RNA effector molecule(s) forms a stem-loop or hairpin

5 dsRNA structure. In some aspects, said double-stranded RNA effector molecule(s) are duplex dsRNAs, formed from two separate RNA strands. In some aspects, the method involves administering to a mammalian cell an expression construct encoding one, two, three, four, five or more of said dsRNA effector molecules. In some embodiments designed to target the HCV minus strand, the dsRNA effector molecule will comprise (a) an RNA sequence corresponding to

10 a hepatitis C virus DNA coding strand sequence as specified above, and (b) the reverse complement of said RNA sequence, optionally linked by a loop sequence. In some embodiments, the dsRNA effector molecule(s) is encoded by an expression construct.

In some aspects the invention relates to a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6 or more double-stranded RNA effector molecules, or a dsRNA expression construct capable of transcribing one, 2, 3, 4, 5, 6 or more of said dsRNA effector molecules in an *in vivo* mammalian cell, each of said dsRNA effector molecules comprising (a) an RNA sequence equivalent to a hepatitis C virus DNA coding strand sequence selected from the group consisting of sequence position 9510-9531,

20 9510-9533, 9510-9534, 9510-9535, 9510-9536, 9514-9534, 9514-9535, 9514-9536, 9514-9539, 9514-9540, 9514-9542, 9517-9539, 9517-9540, 9517-9542, 9517-9544, 9518-9539, 9518-9540, 9518-9542, 9518-9544, 9520-9540, 9520-9542, 9520-9544, 9520-9548, 9521-9542, 9521-9544, 9521-9548, 9521-9549, 9522-9542, 9522-9544, 9522-9548, 9522-9549, 9527-9548, 9527-9549, 9527-9551, 9527-9552, 9527-9553, 9527-9555, 9528-9548, 9528-9549, 9528-9551, 9528-9552,

25 9528-9553, 9528-9555, 9530-9551, 9530-9552, 9530-9553, 9530-9555, 9530-9557, 9530-9558, 9532-9552, 9532-9553, 9532-9555, 9532-9557, 9532-9558, 9532-9559, 9532-9560, 9537-9557, 9537-9558, 9537-9559, 9537-9560, 9537-9561, 9537-9564, 9538-9558, 9538-9559, 9538-9560, 9538-9561, 9538-9564, 9538-9566, 9541-9561, 9541-9564, 9541-9566, 9541-9568, 9541-9569, 9543-9564, 9543-9566, 9543-9568, 9543-9569, 9543-9571, 9545-9566, 9545-9568, 9545-9569,

30 9545-9571, 9545-9573, 9546-9564, 9546-9566, 9546-9569, 9546-9571, 9546-9573, 9547-9568, 9547-9569, 9547-9571, 9547-9573, 9547-9575, 9550-9571, 9550-9573, 9550-9575, 9550-9577,

9550-9578, 9554-9575, 9554-9577, 9554-9578, 9554-9580, 9556-9577, 9556-9578, 9556-9580,
9556-9584, 9562-9584, 9562-9586, 9562-9587, 9562-9588, 9562-9589, 9563-9584, 9563-9586,
9563-9587, 9563-9588, 9563-9589, 9563-9591, 9565-9586, 9565-9587, 9565-9588, 9565-9589,
9565-9591, 9565-9593, 9567-9587, 9567-9588, 9567-9589, 9567-9591, 9567-9593, 9567-9595,

5 9570-9591, 9570-9593, 9570-9595, 9570-9596, 9570-9598, 9572-9593, 9572-9595, 9572-9596,
9572-9598, 9574-9595, 9574-9596, 9574-9598, 9574-9601, 9576-9596, 9576-9598, 9576-9601,
9576-9604, 9579-9601, 9579-9604, 9581-9601, 9581-9604, and 9583-9604 and (b) the reverse
10 complement of said selected RNA sequence equivalent to the hepatitis C virus DNA coding
strand sequence. In some embodiments, said RNA sequences (a) and (b) are linked by a loop
sequence, and the double-stranded RNA effector molecule(s) is a single RNA strand which
forms a stem-loop or hairpin dsRNA structure. In other embodiments, the dsRNA effector
molecule(s) is a duplex dsRNA molecule formed from two separate strands of RNA.

In another aspect, the invention relates to compositions for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an in vivo mammalian cell comprising at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6 or more double-stranded RNA effector molecules, or a dsRNA expression construct capable of expressing one, 2, 3, 4, 5, 6 or more of said dsRNA effector molecules in an in vivo mammalian cell, each of said dsRNA effector molecules comprising (a) a sequence selected from the group consisting of SEQ ID NO: 63; SEQ ID NO: 64; SEQ ID NO: 65; SEQ ID NO: 66; SEQ ID NO: 67, SEQ ID NO: 68, SEQ 20 ID NO: 69; SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73; SEQ ID NO: 74; SEQ ID NO: 75; and SEQ ID NO: 76; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In certain preferred embodiments, the sequence is selected from the group consisting of SEQ ID NO: 72; SEQ ID NO: 73; SEQ ID NO: 74; SEQ ID NO: 75; and SEQ ID NO: 76.

25 In another aspect, the invention relates to methods for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an in vivo mammalian cell comprising administering at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6 or more double-stranded RNA effector molecules, or a dsRNA expression construct capable of expressing one, 2, 3, 4, 5, 6 or more of said dsRNA effector molecules in an in vivo mammalian 30 cell, each of said dsRNA effector molecules comprising (a) a sequence selected from the group consisting of SEQ ID NO: 63; SEQ ID NO: 64; SEQ ID NO: 65; SEQ ID NO: 66; SEQ ID NO:

67, SEQ ID NO: 68, SEQ ID NO: 69; SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO: 73; SEQ ID NO:74; SEQ ID NO:75; and SEQ ID NO:76; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In certain preferred embodiments, the sequence is selected from the group consisting of SEQ ID NO:72; SEQ ID NO: 73; SEQ ID NO:74; SEQ ID NO: 75; and SEQ ID NO:76.

In another aspect, the invention relates to a polynucleotide sequence comprising an RNA sequence equivalent to and/or complementary to a hepatitis C virus DNA coding strand sequence selected from the group consisting of sequence position 9510-9531, 9510-9533, 9510-9534, 9510-9535, 9510-9536, 9514-9534, 9514-9535, 9514-9536, 9514-9539, 9514-9540, 9514-9542, 9517-9539, 9517-9540, 9517-9542, 9517-9544, 9518-9539, 9518-9540, 9518-9542, 9518-9544, 9520-9540, 9520-9542, 9520-9544, 9520-9548, 9521-9542, 9521-9544, 9521-9548, 9521-9549, 9522-9542, 9522-9544, 9522-9548, 9522-9549, 9527-9548, 9527-9549, 9527-9551, 9527-9552, 9527-9553, 9527-9555, 9528-9548, 9528-9549, 9528-9551, 9528-9552, 9528-9553, 9528-9555, 9530-9551, 9530-9552, 9530-9553, 9530-9555, 9530-9557, 9530-9558, 9532-9552, 9532-9553, 9532-9555, 9532-9557, 9532-9558, 9532-9559, 9532-9560, 9537-9557, 9537-9558, 9537-9559, 9537-9560, 9537-9561, 9537-9564, 9538-9558, 9538-9559, 9538-9560, 9538-9561, 9538-9564, 9538-9566, 9541-9561, 9541-9564, 9541-9566, 9541-9568, 9541-9569, 9543-9564, 9543-9566, 9543-9568, 9543-9569, 9543-9571, 9545-9566, 9545-9568, 9545-9569, 9545-9571, 9545-9573, 9546-9564, 9546-9566, 9546-9569, 9546-9571, 9546-9573, 9547-9568, 9547-9569, 9547-9571, 9547-9573, 9547-9575, 9550-9571, 9550-9573, 9550-9575, 9550-9577, 9550-9578, 9554-9575, 9554-9577, 9554-9578, 9554-9580, 9556-9577, 9556-9578, 9556-9580, 9556-9584, 9562-9584, 9562-9586, 9562-9587, 9562-9588, 9562-9589, 9563-9584, 9563-9586, 9563-9587, 9563-9588, 9563-9589, 9563-9591, 9565-9586, 9565-9587, 9565-9588, 9565-9589, 9565-9591, 9565-9593, 9567-9587, 9567-9588, 9567-9589, 9567-9591, 9567-9593, 9567-9595, 9570-9591, 9570-9593, 9570-9595, 9570-9596, 9570-9598, 9572-9593, 9572-9595, 9572-9596, 9572-9598, 9574-9595, 9574-9596, 9574-9598, 9574-9601, 9576-9596, 9576-9598, 9576-9601, 9576-9604, 9579-9601, 9579-9604, 9581-9601, 9581-9604, and 9583-9604.

Applicants' invention further provides a method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell a double-stranded RNA effector molecule comprising at least 19

contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. In a preferred embodiment of the method, effector sequences from more than one SEQ ID sequence may be administered to the same cell, and/or more than one effector sequence from within the same SEQ ID sequence may be administered to the same cell.

5 Applicants further provide a method for inhibiting expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering to said cell a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:27; wherein U is substituted for T. In a preferred embodiment of this aspect of the method, effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 may be administered to the same cell; or from both SEQ ID NO: 11 and SEQ ID NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from each of SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO:27, are administered to the same cell; and/or more than one effector molecule from within the same SEQ ID NO may be administered to the same cell.

10 Applicants further provide a method for inhibiting expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in the same *in vivo* mammalian cell, comprising administering to said cell a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:27; wherein U is substituted for T. In preferred embodiments of this aspect of the invention, effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 may be administered to the same cell; and/or effector molecules from both SEQ ID NO:11 and SEQ ID NO:12; or from both SEQ ID NO: 11 and SEQ ID NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from SEQ ID NO: 11, SEQ ID NO:12 and SEQ ID NO:27; may be administered to the same

cell; and/or more than one effector molecules from within the same SEQ ID NO may be administered to the same cell.

Applicants further provide a composition for inhibiting the expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO is present in the composition.

Applicants further provide a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12 and SEQ ID NO:27; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 are present in the composition; or from both SEQ ID NO: 11 and SEQ ID NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from each of SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO:27, are present in the same composition, and/or wherein more than one effector molecule from within the same SEQ ID NO may be present in the composition.

Applicants further provide a composition for inhibiting the expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in a single *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12 and SEQ ID NO:27; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector

molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12; or from both SEQ ID NO: 11 and SEQ ID NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from each of SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO:27, are present in the composition; and/or wherein more than one effector sequence from within the same SEQ ID NO may be present in the composition.

In particularly preferred embodiments of the above methods and compositions of the invention, the polynucleotide sequence is present within a double-stranded region of an RNA, and the mammalian cell is a human cell.

10 Further provided are compositions for inhibiting the expression of a polynucleotide sequence of hepatitis B virus and/or a polynucleotide sequence of hepatitis C virus in mammalian cells, wherein said compositions comprise an at least 19 contiguous nucleotide sequence selected from within SEQ ID NO:1 through SEQ ID NO:12, and SEQ ID NO:27; the complement sequences of said SEQ ID NO:1 through SEQ ID NO:12, and SEQ ID NO: 27 sequences, and mixtures of these sequences. In this embodiment of the invention, the “an at least 19 contiguous nucleotide sequence” is preferably DNA, and the mammalian cell is preferably human. Also provided are expression constructs comprising any of the aforementioned compositions and a mammalian cell comprising said expression constructs.

20 Another aspect of the invention provides for a polynucleotide sequence comprising a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for polynucleotide sequence comprising nucleotides 1-19, 1-20, 1-21, 2-20, 2-21, or 3-21 of a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for a polynucleotide sequence comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from SEQ ID NO:27 through SEQ ID NO:44.

25 Another aspect provides for a polynucleotide sequence comprising a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for polynucleotide sequence comprising nucleotides 1-19, 1-20, 1-21, 2-20, 2-21, or 3-21 of a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for a polynucleotide sequence comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from SEQ ID NO:27 through SEQ ID NO:44.

~~Another aspect of the invention provides a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in a mammalian cell, comprising a double stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:27; wherein U is substituted for T.~~

- 5 ~~Another aspect provides a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in a mammalian cell, comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:27; wherein U is substituted for T.~~

10 ~~In various aspects of the foregoing methods and compositions, the in vivo mammalian cell is an in vivo human cell.~~

BRIEF DESCRIPTION OF THE SEQUENCES

- SEQ ID NO:1 through SEQ ID NO:10 represent conserved regions of the hepatitis B genome.
SEQ ID NO:11 and SEQ ID NO:12 represent conserved regions of the hepatitis C genome.
15 SEQ ID NO:13 represents the nucleotide sequence of human U6 promoter.
SEQ ID NO:14 and SEQ ID NO:15 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:5.
SEQ ID NO:16 and SEQ ID NO:17 represent eiRNAs that have HBV 20 sequences mapping within SEQ ID NO:4.
20 SEQ ID NO:18 represents eiRNA that has an HBV sequence mapping within SEQ ID NO:10.
SEQ ID NO:19 through SEQ ID NO:22 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:3.
SEQ ID NO:23 and SEQ. ID NO:24 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:2.
25 SEQ ID NO:25 and SEQ ID NO:26 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:1.
SEQ ID NO:27 represents the “X” region of the HCV 3’UTR.
SEQ ID NO:28 through SEQ ID NO:36 represent siRNAs mapping to the the HCV 3’UTR.

SEQ ID NO:37 through SEQ ID NO:44 represent siRNAs mapping to the “X” region of the HCV 3’UTR.

SEQ ID NO:45 represents an siRNA mapping to the HCV core.

SEQ ID NO:46 represents an siRNA mapping to lamin.

5 SEQ ID NO:47 represents the T7 RNA polymerase gene.

~~SEQ ID NO:48 represents a T7 based eiRNA vector encoding a hairpin RNA.~~

SEQ ID NO:48 represents a 5’ segment of the hepatitis C virus sequence (corresponds to positions 36 to 358 in Genbank Accession Number AJ238799, with 2 base changes, C to G at AJ238799 position 204 and G to A at AJ238799 position 357).

10 SEQ ID NO:49 represents an eiRNA (shRNA) molecule to a conserved HBV sequence.

SEQ ID NO:50 through SEQ ID NO:62 represent the first 21 nucleotides of SEQ ID NOs: 14-23, 25-26, and 49.

SEQ ID NO:63 through SEQ ID NO:71 represent the first 21 nucleotides of SEQ ID NOs: 28-36.

15 SEQ ID NO:72 through SEQ ID NO:76 represent highly conserved coding region sequitopes from the 5’ and 3’ untranslated regions of HCV.

SEQ ID NO:77 through SEQ ID NO:109 represent highly conserved HCV sequences from the 5’ UTR of the HCV (SEQ ID NO: 11).

BRIEF DESCRIPTION OF THE DRAWINGS

20 Figure 1 depicts a vector illustrating placement of the T7 RNA polymerase promoter and T7 RNA polymerase, showing inclusion of hairpin eiRNA sequences.

Figure 2 is a graph showing HBsAg inhibition corresponding to data 15 found in Table 2.

Figure 3 is a graph showing HBsAg inhibition corresponding to data found in Table 3.

Figure 4 is a graph showing HBsAg inhibition corresponding to data found in Table 4.

25 Figure 5 is a graph showing HBsAg inhibition corresponding to data found in Table 5.

Figure 6 is a graph showing HBsAg inhibition corresponding to data found in Table 6.

Figure 7 is a graph showing HBsAg inhibition corresponding to data found in Table 7.

Figure 8 is a graph showing HBsAg inhibition corresponding to data found in Table 8.

Figure 9 is a drawing depicting effective HBV-AYW shRNA inserts.

Figure 10 is a graph showing HBsAg inhibition corresponding to data found in Table 9.

5 Figure 11 is a bar graph showing downregulation of HBV RNA by Northern Blot analysis.

Figure 12 is a graph showing HBsAg inhibition corresponding to data found in Table 12.

10 Figure 13 is a Western Blot showing levels of HCV NS5A protein at (l to r) 0, 9, and 20 pmole of the identified siRNAs, as described in more detail in Experiment 1 of Example 2.

Figure 14 is a Western Blot showing levels of HCV NS5A protein at 20 (l to r) 0, 9, and 20 pmole of the identified siRNA, and 0, 3, and 9 pmole of the “core” positive control siRNA, as described in more detail in Experiment 2 of Example 2.

15 Figure 15 is a table of additional conserved HCV genome sequence segments suitable for generating dsRNA effector molecules which inhibit the expression of polynucleotide sequences of hepatitis C virus, including expressed shRNA for gene silencing. Each sequence represents a DNA coding strand sequence in standard 5' to 3' polarity which (together with its reverse complement) can be utilized to transcribe or design a double-stranded RNA effector molecule, e.g., an shRNA or duplex dsRNA molecule targeted to degrade the negative strand of HCV
20 RNA. E.g., an DNA sequence, followed by a loop sequence (e.g., a 9 base loop sequence as described elsewhere herein), followed by the reverse complement of the sequence given in the table, may be incorporated into an expression construct under the control of an appropriate promoter. The shRNA molecule transcribed from such an expression construct is expected to inhibit expression of HCV polynucleotide sequences and/or mediate dsRNA silencing of HCV.
25 For example, in the case of the 22 base sequence shown for positions 9545-9566, a construct is made to contain a 53 bp insert, comprising the 22 base sequence of 9545-9566, a linker or loop

sequence, and the reverse complement of the 9545-9566 sequence, preferably under the control of an RNA polymerase III promoter and ending with an RNA polymerase III terminator, e.g., a run of 4, 5, or more T residues. The RNA equivalent of this sequence, having U's instead of T's, would read (in the 5' to 3' direction):

5 AAAGGUCCGUGAGCCGCUUGAC-XXXXXXXXX-
GUCAAGCGGUCACGGACCUU U

where X represents bases of the loop that are unable to form stable base pairs with any other portion of the 53 by shRNA sequence. The loop may vary considerably, however, as to both length and nucleotide sequence, so long as the formation of the double-stranded "stem" 10 region of the hairpin is not adversely affected. Thus, in expression constructs that are the subject of this invention, the sequence element above beginning at the end which reads 5' AAAGGT is cloned into an appropriate vector downstream from and operably linked to the promoter. As described elsewhere herein, in preferred embodiments, two, three, four, five, six, seven, or more of the shRNAs encoded by these sequences, optionally, together with other anti-HCV, and/or 15 HBV sequences described herein, are coded into and expressed by a single dsRNA expression vector. In one aspect, each of said multiple stem-loop or shRNA molecules is encoded in a single expression vector within a different expression cassette, each operably linked to a promoter and a terminator, preferably a polymerase III promoter, which may be the same or different. In another aspect, two or more hairpin dsRNA molecules may be expressed from a 20 single promoter, as e.g., a bi-fingered molecule in which a single transcribed RNA strand comprises two such shRNA sequences separated by an unrelated linker sequence. Such constructs, in which a single expression vector provides a mammalian cell with two, three, four, five or more independent dsRNA effector molecules against an HCV and/or HBV target polynucleotide, are particularly desirable for pharmaceutical applications. An alternative means 25 of dsRNA-mediated silencing may be accomplished by preparing shRNAs or duplex dsRNAs corresponding to the identified sequences by chemical synthesis or in vitro expression and delivering them into a cell in order to achieve inhibition of HCV and/or HBV polynucleotide sequences.

DETAILED DESCRIPTION OF THE INVENTION

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing or transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Since RNA interference acts in a sequence specific manner, the RNAi molecule used as a drug must be specific to its target. Viral genomes are variable to accommodate resistance to changes in the environment. While HBV and HCV are very desirable viral targets for RNAi, the variability and mutability of the viruses and the high rates of transcription of the viruses make HBV and HCV very challenging targets for any therapeutic and/or prophylactic approach. In order to knock down viral genome replication using RNAi there is a need to identify conserved and unique regions in the viral genome. At that same time, it is very important in order to avoid toxicity that any sequences selected for gene silencing be absent from the human genome.

Human Hepatitis B Virus (HBV) Hepatitis B virus belongs to the family of hepadnaviruses. The HBV genome is a relaxed circular, partially double stranded DNA of approximately 3,200 base pairs. There are 4 partially overlapping open reading frames encoding the envelope (pre-S/S), core (precore/core), polymerase, and X proteins. The pre-S/S open reading frames encode the large (L), middle (M), and small (S) surface glycoproteins. The precore/core open reading frame is translated into a precore polypeptide, which is modified into a soluble protein, the hepatitis B e antigen (HBeAg) and the nucleocapsid protein, hepatitis B core antigen. Mutations in the core promoter and precore region have been shown to decrease or abolish HBeAg production. The polymerase protein functions as a reverse transcriptase as well as a DNA polymerase. The X protein is a potent transactivator and may play a role in hepatocarcinogenesis.

The replication cycle of HBV begins with the attachment of the virion to the hepatocyte. Inside the hepatocyte nucleus, synthesis of the plus strand HBV DNA is completed and the viral genome is converted into a covalently closed circular DNA (cccDNA). Most antiviral agents that have been examined so far have little or no effect on cccDNA, which accounts for the rapid reappearance of serum HBV DNA after cessation of antiviral therapy. The aims of treatment of chronic hepatitis B are to achieve sustained suppression of HBV replication and/or expression of HBV antigens and remission of liver disease.

In GenBank version 132.0 there are more than 4500 HBV sequences and 340 HBV complete genome sequences (317 Human isolates, 22 isolates from other primates and one woodchuck HBV isolate). This variability constitutes a serious challenge for sequence-specific pharmaceutical approaches such as RNAi. In order to identify conserved sequences suitable for RNAi applications, a comparison between all the complete genomes was carried out using a modified version of ClustalW. Two multiple alignment schemes were generated: the first included all 339 HBV complete genome sequences and the second was limited to all Human HBV isolates. The multiple alignment results were parsed and a table that included scores for sequence conservation at each position in the HBV genome was generated. A sliding window search to identify the longest region of sequence conservation larger than 19 nt in length was created. Three major conserved regions were identified and mapped to GenBank accession no.: AF090840, a Human HBV isolate. The conserved HBV sequences were screened against Genbank sequences of both human genomic and cDNA libraries (Human chromosomes database). It was found that the identified conserved viral sequences are unique from 21 nt up.

For human therapeutic purposes, assuring that homologous human sequences are not inadvertently silenced is as important as selecting conserved viral sequences for RNAi.

In GenBank version 132.0 there are more than 4500 HBV sequences and 340 HBV complete genome sequences (317 Human isolates, 22 isolates from other primates and one woodchuck HBV isolate). This variability constitutes a serious challenge for sequence-specific pharmaceutical approaches such as RNAi. In order to identify conserved sequences suitable for RNAi applications, a comparison between all the complete genomes was carried out using a modified version of ClustalW. Two multiple alignment schemes were generated: the first included all 339 HBV complete genome sequences and the second was limited to all Human HBV isolates. The multiple alignment results were parsed and a table that included scores for sequence conservation at each position in the HBV genome was generated. A sliding window search to identify the longest region of sequence conservation larger than 19 nt in length was created. Three major conserved regions were identified and mapped to GenBank accession no.: AF090840, a Human HBV isolate. The conserved HBV sequences were screened against Genbank sequences of both human genomic and cDNA libraries (Human chromosomes database). It was found that 21 nucleotide and longer segments selected as a permuted "window" from within the conserved regions were unique to HBV, i.e. no perfect sequence

matches exist between any 21 nt or longer HBV conserved segments and the available sequence databases of human chromosomal and RNA sequences. For human therapeutic purposes, assuring that homologous human sequences are not inadvertently silenced is as important as selecting conserved viral sequences for RNAi.

5 Human Hepatitis C Virus HCV is a small (40 to 60 nanometers in diameter), enveloped, single-stranded RNA virus of the family Flaviviridae and genus hepacivirus. The genome is approximately 10,000 nucleotides and encodes a single polyprotein of about 3,000 amino acids, which is post-transcriptionally cleaved into 10 polypeptides, including 3 major structural (C, E1, and E2) and multiple non-structural proteins ([NS] NS2 to NS5). The NS proteins include
10 enzymes necessary for protein processing (proteases) and viral replication (RNA polymerase). Because the virus mutates rapidly, changes in the envelope proteins may help it evade the immune system. There are at least 6 major genotypes and more than 90 subtypes of HCV. The different genotypes have different geographic distributions. Genotypes 1a and 1b are the most common in the United States (about 75 % of cases). Genotypes 2a and 2b (approximately 15%)
15 and 3 (approximately 7%) are less common.

There is little difference in the severity of disease or outcome of patients infected with different genotypes. However, patients with genotypes 2 and 3 are more likely to respond to interferon treatment. The virus replicates at a high rate in the liver and has marked sequence heterogeneity. The main goal of treatment of chronic hepatitis C is to eliminate detectable viral
20 RNA from the blood. Lack of detectable hepatitis C virus RNA from blood six months after completing therapy is known as a sustained response. Studies suggest that a sustained response is equated with a very favorable prognosis and that it may be equivalent to a cure. There may be other more subtle benefits of treatment, such as slowing the progression of liver scarring (fibrosis) in patients who do not achieve a sustained response.

25 In GenBank version 134.0 there are more than 20,000 HCV sequences and 93 HCV complete genome sequences. A comparison between all the complete genomes was carried out using a modified version of ClustalW. The multiple alignment result was parsed and a table that included scores for sequence conservation at each position in the HCV genome was generated. A sliding window search to identify the longest region of sequence conservation larger than 19 nt
30 in length was created. Three major conserved regions were identified and mapped to GenBank RefSeq (reference sequence) accession no.: NC_004102 this is GenBank annotated HCV

~~complete genome. The conserved sequences were screened against Genbank sequences of both human genomic and cDNA libraries (human chromosomes database), and those sequences unique from 21 nt up were identified.~~

In GenBank version 134.0 there are more than 20,000 HCV sequences and 93 HCV complete genome sequences. A comparison between all the complete genomes was carried out using a modified version of ClustalW. The multiple alignment result was parsed and a table that included scores for sequence conservation at each position in the HCV genome was generated. A sliding window search to identify the longest region of sequence conservation larger than 19 nt in length was created. Three major conserved regions were identified and mapped to GenBank RefSeq (reference sequence) accession no.: NC_004102 this is GenBank annotated HCV complete genome. The three major conserved regions include a portion of the 3' untranslated region of the virus, already described in the literature to be well-conserved among viral isolates. See, e.g., US Patent 5,874,565, "Nucleic Acids Comprising a Highly Conserved Novel 3' Terminal Sequence Element of the Hepatitis C Virus." However, the instant disclosure represents a comprehensive and detailed analysis of these conserved regions to the extent that permitted the discovery and evaluation of multiple short segments suitable for use alone and in combination as a therapeutic for silencing HCV among a diverse patient population. The conserved sequences were screened against Genbank sequences of both human genomic and cDNA libraries (human chromosomes database), and the series of permuted HCV segments greater than 20 bases long with no homology to the human sequence databases were identified.

Non-Homology with Human Sequences

It is equally important to ensure that conserved viral sequences targeted for silencing according to the invention be substantially non-homologous to any naturally occurring, normally functioning, and essential human polynucleotide sequence, so that the dsRNA molecule does not adversely affect the function of any essential naturally occurring mammalian polynucleotide sequence, when used in the methods of this invention. Such naturally occurring functional mammalian polynucleotide sequences include mammalian sequences that encode desired proteins, as well as mammalian sequences that are non-coding, but that provide for essential regulatory sequences in a healthy mammal. Essentially, the RNA molecule useful in this invention must be sufficiently distinct in sequence from any mammalian polynucleotide

sequence for which the function is intended to be undisturbed after any of the methods of this invention are performed. Computer algorithms may be used to define the essential lack of homology between the RNA molecule polynucleotide sequence and the normal mammalian sequences.

5 ~~Since the length of a contiguous dsRNA sequence capable of association with and activation of RISC (RNA induced silencing complex), is generally considered to be 19-27 base pairs, the identified conserved HBV and HCV sequences were compared with both human genomic libraries and, perhaps even more importantly, with human cDNA libraries. Since human cDNA libraries represent expressed sequences that appear in mRNAs, such mRNA sequences would be especially vulnerable to silencing by homologous dsRNA sequences provided to a cell.~~

10 ~~Since the length of a contiguous dsRNA sequence capable of association with and activation of RISC (RNA-induced silencing complex), is generally considered to be 19-27 base pairs, the identified conserved HBV and HCV sequences were compared with both human genomic libraries and, perhaps even more importantly, with human cDNA libraries as described above. Since human cDNA libraries represent expressed sequences that appear in mRNAs, such mRNA sequences would be especially vulnerable to silencing by homologous dsRNA sequences provided to a cell.~~

15 Accordingly, the conserved HBV and HCV sequences were compared with human genomic and cDNA sequences. No human cDNA library matches to the HBV or HCV conserved sequences were identified. (Although there were some matches that were ultimately identified as HBV contamination in the cDNA library.) A comparison with human genomic library sequences revealed no match of any sequence of 21 nts or more, one match of 20 nucleotides, and one match of 19 nucleotides. These matches were in non-coding regions, and 20 likely do not appear in mRNA since cognates were not turned up in the cDNA library. Therefore, they are considered unlikely to be a safety risk, but could be excluded if desired.

Conserved sequences from HBV and HCV

HBV Conserved Region 1

25 GAACATGGAGA[A(89%)/G(11%)]CA[T(76%)/C(24%)][C(78%)/A(20%)/T(2%)][A(7 8%)/G(21%)/T(1%)]CATCAGGA[T(65%)/c(35%)]TCCTAGGACCCCTGCTCGTTACAG GCGG[G(88%)/t(12%)]GT[T(89%)/G(11%)]TTTCT[T(94%)/C(6%)]GTTGACAA[G(64%)/A(

36%)]AATCCTCACAAATACC[A(56%)/G(43%)/T(1%)]CAGAGTCTAGACTCGTGGTGGAC
TTCTCTCAATTCTAGGGG[G(92%)/A(5%)/T(3%)]A[A(41%)/G(30%)/T(18%)/C(11%)][C
(90%)/T(10%)]

HBV Conserved Region 2

5 TGGATGTGTCT[G(99%)/A(1%)]CGGCGTTTATCAT

HBV Conserved Region 3

AAGGCCTTCT[A(43%)/G(43%)/C(14%)][T(56%)/A(37%)/C(7%)]GT[A(87%)/C(13
%)]AAC[A(57%)/G(43%)]TA[T(59%)/C(41%)][C(59%)/A(41%)]TG[A(92%)/C(8%)][A(93%
%)/C(7%)]CaTTTACCCCGTTGC[T(54%)/C(46%)][C(92%)/A(8%)]GGCAACGG[C(74%)/T(24
%)C[A(50%)/T(43%)/c(7%)]GG[T(87%)/C(13%)]CT[G(70%)/C(19%)/T(7%)/A(4%)]TGCCA
10 AGTGTGCTGACGCAACCCCCACTGG[C(48%)/T(38%)/A(14%)]TGGGGCTTGG[C(84
%)/T(16%)][C(84%)/T(12%)/G(4%)]AT[A(47%)/T(23%)/G(17%)/C(13%)]GGCCATC[A(83%
/G(17%)][G(92%)/C(8%)]CGCATGCGTGGAACCTT[G(84%)/C(13%)/T(3%)][T(92%)/A(4
%)/C(3%)/G(1%)]G[G(78%)/T(22%)]CTCCTCTGCCGATCCATACTGCGGAACTCCT[A(88
%)
15 /T(9%)/G(1%)/C(1%)]GC[C(57%)/A(35%)/T(6%)/G(2%)]GC[T(92%)/C(7%)/G(1%)]TGTT
T[T(88%)/C(12%)]GCTCGCAGC[C(64%)/A(36%)]GGTCTGG[A(87%)/G(13%)]GC

HBV Conserved Region 4

[C(62%)/T(38%)]ACTGTTCAAGCCTCAAGCTGTGCCTGGGTGGCTTT[G(88%)/A
(12%)]GG[G(92%)/A(8%)]CATGGACATTGAC[C(92%)/A(8%)]C[T(65%)/G(35%)]TATAAA
20 GAATTGGAGCT[A(65%)/T(35%)]CTGTGGAGTTACTCTC[G(62%)/T(35%)/A(3%)]TTTT
TGCCTTC[T(92%)/C(8%)]GACTT[C(92%)/T(8%)]TTTCCTTC

HBV Conserved Region 5

{C(69%)/del(31%)}{G(69%)/del(31%)}A[G(85%)/T(11%)/C(4%)]GCAGGTCCCCTAG
AAGAAGAACTCCCTCGCCTCGCAGACG[C(61%)/A(39%)]G[A(62%)/G(38%)]TCTCAAT
25 CG[C(88%)/A(12%)]CGCGTCGCAGAAGATCTCAAT[C(92%)/T(8%)]TCGGGAATCT[C(88
%)/T(12%)]AATGTTAGTAT

HBV Conserved Region 5

[C(69%)/del(31%)1[G(69%)/del(31%)]A[G(85%)/T(11%)/C(4%)]GCAGG
TCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACG[C(61%)/A(39%)1AG[A(62%)/
G(38%)]TCTCAATCG[C(88%)/A(12%)]CGCGTCGCAGAACGATCTCAAT[C(92%)/T(8
5 %)]TCGGGAATCT[C(88%)/T(12%)]AATGTTAGTAT

HBV Conserved Region 6

TTGG[C(84%)/t(16%)][C(84%)/t(12%)/g(4%)]AT[A(47%)/t(23%)/g(17%)/c(13%)]GG
CCATC[A(83%)/g(17%)][G(92%)/c(8%)]CGCATGCGTGGAACCTTT[G(84%)/c(13%)/t(3%)]
10 [T(92%)/a(4%)/c(3%)/g(1%)]G[G(78%)/t(22%)]CTCCTCTGCCGATCCATACTGCGGAACCT
CCT[A(88%)/t(9%)/g(1%)/c(1%)]GC[C(57%)/a(35%)/t(6%)/g(2%)]GC[T(92%)/c(7%)/g(1%)]T
GTTC[T(88%)/c(12%)]GCTCGCAGC[C(64%)/a(36%)]GGTCTGG[A(87%)/g(13%)]GC

HBV Conserved Region 7

CTGCCAACTGGAT[C(86%)/T(10%)/A(4%)]CT[C(69%)/T(25%)/A(6%)]CGCGGGAA
15 CGTCCTTGT[T(75%)/C(25%)]TACGTCCCCGTC[G(93%)/A(7%)]GCGCTGAATCC[C(86%)/
T(7%)/A(7%)]GCGGACGACCC[C(52%)/G(25%)/T(19%)/A(4%)]

HCV Conserved Region 1

[A(74%)/G(19%)/T(7%)][G(82%)/A(15%)/T(3%)]ATCACTCCCCTGTGAGGAACTA
CTGTCTCACGCAGAAAGCGTAGCCATGGCGTTAGTATGAGTGT[C(92%)/T(7%)]G
20 TGCAGC[C(89%)/T(10%)]TCCAGG[A(76%)/T(14%)/C(8%)/G(1%)]CCCCCCCTCCCGGGA
GAGCCATAGTGGTCTGCCAACCGGTGAGTACACCGGAATTGCC[A(90%)/G(9%)]GG
A[C(78%)/T(16%)/A(5%)]GACCGGGTCCTTCTTGGAT[G(78%)/T(11%)/A(10%)]AACCC
GCTC[A(94%)/T(5%)]ATGCC[T(90%)/C(9%)]GGA[G(91%)/C(4%)/A(4%)]ATTGGGGCGTG
CCCCCGC[G(85%)/A(14%)]AGAC[T(94%)/C(5%)]GCTAGCCGAGTAG[T(92%)/C(7%)]GT
25 TGGGT[C(94%)/T(5%)]GCGAAAGGCCTGTGGTACTGCCTGATAGGGTGCTGCGAGT
GCCCGGGAGGTCTCGTAGACCGTGCA[C(62%)/T(30%)/A(8%)]CATGAGCAC[A(50%)/
G(50%)][A(92%)/C(8%)][A(89%)/T(11%)]TCC[T(92%)/A(5%)/C(3%)]AAACC[T(84%)/C(14
%)/A(2%)]CAAAGAAAAACCAAA[C(84%)/A(16%)]G[T(84%)/A(16%)]AACACCAACCG[
C(77%)/T(23%)]GCCAACAGGACGT[C(81%)/T(18%)/A(1%)]AAGTMCCGGG[C(89%)/T(11%)]GG[T(80%)/C(20%)]GG[T(80%)/C(17%)/A(3%)]CAGATCGTTGG[T(91%)/C(8%)/G(1

%)]GGAGT[T(87%)/A(11%)/C(2%)]TAC[C(74%)/T(20%)/G(6%)]TGTTGCCGCGCAGGGG
 CCC[C(87%)/T(8%)/A(4%)/G(1%)][A(92%)/C(8%)][G(92%)/A(5%)/C(2%)][G(87%)/A(12%)/
 T(1%)]TTGGGTGTGCGCGCAGAC[T(78%)/G(13%)/A(7%)/C(2%)]AGGAAGACTTC[C(90%)
 /G(5%)/T(5%)]GA[G(90%)/A(10%)]CGGTC[G(79%)/C(12%)/A(8%)/T(1%)]CA[A(86%)/G(14
 5 %)]CC[T(88%)/A(6%)/C(6%)]CG[T(82%)/C(9%)/A(9%)]GG[A(87%)/T(8%)/G(3%)/C(2%)]AG

HCV Conserved Region 2

ATGGC[T(76%)/A(12%)/C(10%)/G(2%)]TGGGATATGATGATGAACCTGG[T(81%)/C
 (19%)]C

Conserved Consensus Sequences presented in SEQ ID format

10 The following sequences are presented in the format required per the WIPO Standard
 ST.25 (1998), using the codes provided under 37 CFR 1.821. SEQ ID NO:1 through SEQ ID
 NO:10 are derived from the HBV genome SEQ ID NO:11 and SEQ ID NO:12 are derived from
 the HCV genome.

SEQ ID NO:1 HBV

15 GAACATGGAGArCAyhdCATCAGGAyTCCTAGGACCCCTGCTCGTACAGGC
 GGkGTkTTTCTyCTTGACAArAATCCTCACAAATACCdCAGAGTCTAGACTCGTGGTGGAA
 CTTCTCTCAATTCTAGGGGdAny

EQ ID NO:2 HBV

TGGATGTGTCTrCGGCGTTITATCAT

20 SEQ ID NO:3 HBV

AAGGCCTTCTvhGTmAACArTAymTGmmCCTTACCCC
 GTTGCymGGCAACGGye hGGyCTnTGCCAAGTGTGCTGACGCAACCCCCACTGG
 hTGGGGCTTGGybATnGGCCATCrsCGCATGCGTGGAA
 CCTTTbnGkCTCCTCTGCCGATCCATACTGCGGAACCTCCTnGCnGCbT
 25 GTTTyGCTCGCAGCmGGTCTGGGrGC

SEQ ID NO:4 HBV

yACTGTTCAAGCCTCAAGCTGTGCCCTGGGTGGCTTTrG
 GrCATGGACATTGACmCkTATAAAGAATTGGAGCTwCTGTGGAGTTACTCTCdTTT
 TGCCTTCyGACTTyTTCCCTTC

SEQ ID NO:5 HBV

5 CGAbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCCTCG
 CAGACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGA
 ATCTyAATGTTAGTAT

SEQ ID NO:6 HBV

10 GACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAAT
 CTyAATGTTAGTAT

SEQ ID NO:7 HBV

15 CAbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCCTCG
 AGACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAAT
 CTyAATGTTAGTAT

SEQ ID NO:8 HBV

GA~~b~~GCAGGTCCCCTAGAAGAAGAACTCCCTGCCCTCG
 AGACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGA
 TCTyAATGTTAGTAT

20 SEQ ID NO:5 HBV

CGAbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCCTCGCAGACG
 mAGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTA
GTAT

25 SEQ ID NO:6 HBV

AbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCCTCGCAGACGmA
 GrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTAGTAT

SEQ ID NO:7 HBVCAbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCTCGCAGACGmAGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTAGTAT5 SEQ ID NO:8 HBVGAbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCTCGCAGACGmAGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTAGTAT

SEQ ID NO:9 HBV

10 TTGGybATnGCCATCrsCGCATGCGTGGAACCTTbnGk

CTCCTCTGCCGATCCATACTGCGGAACCTCTnG

CnGCbTGTTTyGCTCGCAGCmGGTCTGGGrGC

SEQ ID NO:10

HBV CTGCCAACTGGAThCThCGCGGACGTCTTGTyTACG

15 TCCC GTCrGCGCTGAATCChGCGGACGACCCn

SEQ ID NO:11 HCV

DdATCACTCCCCTGTGAGGAACTACTGTCTCACGCAGA

AAGCGTCTAGCCATGGCGTTAGTATGAGTGTyGTGCAGCyTCCAGGn

CCCCCCCCTCCCAGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTA

20 CACCGGAATTGCCrGGAhGACCGGGTCCTTCTGGATdAACCGCT

CwATGCCyGGAvATTGGCGTGCCCCCGCrAGACyGCTAGCCGAGT

AGyGTTGGGTyGCGAAAGGCCTTGTGGTACTGCCTGATAAGGTGCTGCGAGTGC CC

CGGGAGGTCTCGTAGACCGTGCAhCATGAGCACrmwTCChAACChCAAAGAAAAAC

CAAAmGwAACACCAACCGyCGCC

25 CACAGGACGThAAGTTCCCGGGyGGyGGhCAGATCGTTGGbGGAGThTAC

bTGTTGCCGCGCAGGGGCCNmvdTTGGGTGTGCGCGACnAGGA

AGACTTCbGArCGGTCnCArCChCGhGGnAG

*Double Stranded RNA Gene Silencing/RNAi By “nucleic acid composition” or
 “nucleotide” composition is meant any one or more compounds in which one or more molecules

of phosphoric acid are combined with a carbohydrate (e.g., pentose or hexose) which are in turn combined with bases derived from purine (e.g., adenine) and from pyrimidine (e.g., thymine).

Particular naturally occurring nucleic acid molecules include genomic deoxyribonucleic acid (DNA) and host ribonucleic acid (RNA), as well as the several different forms of the latter, e.g.,

- 5 messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Also included are different DNA molecules which are complementary (cDNA) to the different RNA molecules. Synthesized DNA or a hybrid thereof with naturally occurring DNA, as well as DNA/RNA hybrids, and peptide nucleic acid (PNA) molecules (Gambari, Curr Pharm Des 2001 Nov;7(17):1839-62) can also be used.

10 It is contemplated that where the desired nucleic acid molecule is RNA, the T (thymine) in the sequences provided herein is substituted with U (uracil). For example, SEQ ID NO:1 through SEQ ID NO:44 are disclosed herein as DNA sequences. It will be obvious to one of ordinary skill in the art that an RNA effector molecule comprising sequences from any of the aforementioned SEQ ID NOs will have T substituted with U.

15 Nucleic acids typically have a sequence of two or more covalently bonded naturally-occurring or modified deoxyribonucleotides or ribonucleotides. Modified nucleic acids include, e.g., peptide nucleic acids and nucleotides with unnatural bases.

~~By "dsRNA" is meant a nucleic acid containing a region of two or more nucleotides that are in a double stranded conformation. It is envisioned that the conserved viral sequences of the invention may be utilized in any of the many compositions known in the art or subsequently developed which act through a dsRNA mediated gene silencing or RNAi mechanism. In various embodiments, the dsRNA consists entirely of ribonucleotides or consists of a mixture of ribonucleotides and deoxynucleotides, such as the RNA/DNA hybrids disclosed, for example, by WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. The dsRNA may be a single molecule with a region of self complementarity such that nucleotides in one segment of the molecule base pair with nucleotides in another segment of the molecule. In various embodiments, a dsRNA that consists of a single molecule consists entirely of ribonucleotides or includes a region of ribonucleotides that is complementary to a region of deoxyribonucleotides. Alternatively, the dsRNA may include two different strands that have a region of complementarity to each other. In various embodiments, both strands consist entirely of ribonucleotides, one strand consists entirely of ribonucleotides and one strand consists entirely of deoxyribonucleotides.~~

of deoxyribonucleotides, or one or both strands contain a mixture of ribonucleotides and deoxyribonucleotides. Desirably, the regions of complementarity are at least 70, 80, 90, 95, 98, or 100% complementary. Desirably, the region of the dsRNA that is present in a double stranded conformation includes at least 19, 20, 21, 22, 23, 24, 30, 50, 75, 100, 200, 500, 1000, 2000 or 5000 nucleotides or includes all of the nucleotides in a cDNA being represented in the dsRNA. In some embodiments, the dsRNA does not contain any single stranded regions, such as single stranded ends, or the dsRNA is a hairpin. In other embodiments, the dsRNA has one or more single stranded regions or overhangs. Desirable RNA/DNA hybrids include a DNA strand or region that is an antisense strand or region (e.g., has at least 70, 80, 90, 95, 98, or 100% complementarity to a target nucleic acid) and an RNA strand or region that is a sense strand or region (e.g., has at least 70, 80, 90, 95, 98, or 100% identity to a target nucleic acid). In various embodiments, the RNA/DNA hybrid is made *in vitro* using enzymatic or chemical synthetic methods such as those described herein or those described in WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. In other embodiments, a DNA strand synthesized *in vitro* is complexed with an RNA strand made *in vivo* or *in vitro* before, after, or concurrent with the transformation of the DNA strand into the cell. In yet other embodiments, the dsRNA is a single circular nucleic acid containing a sense and an antisense region, or the dsRNA includes a circular nucleic acid and either a second circular nucleic acid or a linear nucleic acid (see, for example, WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999.)

Exemplary circular nucleic acids include lariat structures in which the free 5' phosphoryl group of a nucleotide becomes linked to the 2' hydroxyl group of another nucleotide in a loop back fashion.

In other embodiments, the dsRNA includes one or more modified nucleotides in which the 2' position in the sugar contains a halogen (such as fluorine group) or contains an alkoxy group (such as a methoxy group) which increases the half life of the dsRNA *in vitro* or *in vivo* compared to the corresponding dsRNA in which the corresponding 2' position contains a hydrogen or an hydroxyl group. In yet other embodiments, the dsRNA includes one or more linkages between adjacent nucleotides other than a naturally occurring phosphodiester linkage. Examples of such linkages include phosphoramido, phosphorothioate, and phosphorodithioate linkages. The dsRNAs may also be chemically modified nucleic acid molecules as taught in U.S. Patent No. 6,673,661. In other embodiments, the dsRNA contains one or two capped

strands, as disclosed, for example, by WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. In other embodiments, the dsRNA contains coding sequence or non coding sequence, for example, a regulatory sequence (e.g., a transcription factor binding site, a promoter, or a 5' or 3' untranslated region (UTR) of an mRNA). Additionally, the dsRNA 5 can be any of the at least partially dsRNA molecules disclosed in WO 00/63364, filed April 19, 2000 (see, for example, pages 8-22), as well as any of the dsRNA molecules described in US Provisional Application 60/399,998 filed July 31, 2002, and PCT/US2003/024028, filed 31 Jul 2003; and US Provisional Application 60/419,532 filed October 18, 2002, and PCT/US2003/033466, filed 20 Oct 2003, the teaching of which is hereby incorporated by 10 reference. Any of the dsRNAs may be expressed *in vitro* or *in vivo* using the methods described herein or standard methods, such as those described in WO 00/63364, filed April 19, 2000 (see, for example, pages 16-22).

dsRNA "Hairpin" Constructs: Constructs encoding a unimolecular hairpin dsRNA are more desirable for some applications than constructs encoding duplex dsRNA (i.e., dsRNA 15 composed of one RNA molecule with a sense region and a separate RNA molecule with an antisense region) because the single stranded RNA with inverted repeat sequences more efficiently forms a dsRNA hairpin structure. This greater efficiency is due in part to the occurrence of transcriptional interference arising in vectors containing converging promoters that generate duplex dsRNA. Transcriptional interference results in the incomplete synthesis of each 20 RNA strand thereby reducing the number of complete sense and antisense strands that can base pair with each other and form duplexes. Transcriptional interference can be overcome, if desired, through the use of (i) a two vector system in which one vector encodes the sense RNA and the second vector encodes the antisense RNA, (ii) a bicistronic vector in which the individual strands are encoded by the same plasmid but through the use of separate cistrons, or 25 (iii) a single promoter vector that encodes a hairpin dsRNA, i.e., an RNA in which the sense and antisense sequences are encoded within the same RNA molecule. Hairpin expressing vectors have some advantages relative to the duplex vectors. For example, in vectors that encode a duplex RNA, the RNA strands need to find and base pair with their complementary counterparts soon after transcription. If this hybridization does not happen, the individual RNA strands 30 diffuse away from the transcription template and the local concentration of sense strands with respect to antisense strands is decreased. This effect is greater for RNA that is transcribed

intracellularly compared to RNA transcribed *in vitro* due to the lower levels of template per cell. Moreover, RNA folds by nearest neighbor rules, resulting in RNA molecules that are folded co transcriptionally (i.e., folded as they are transcribed). Some percentage of completed RNA transcripts is therefore unavailable for base pairing with a complementary second RNA because of intra-molecular base pairing in these molecules. The percentage of such unavailable molecules increases with time following their transcription. These molecules may never form a duplex because they are already in a stably folded structure. In a hairpin RNA, an RNA sequence is always in close physical proximity to its complementary RNA. Since RNA structure is not static, as the RNA transiently unfolds, its complementary sequence is immediately available and can participate in base pairing because it is so close. Once formed, the hairpin structure is predicted to be more stable than the original non hairpin structure. Especially desirable are, e.g., "forced" hairpin constructs, partial hairpins capable of being extended by RNA dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31 Jul 2002; and PCT/US2003/024028, "Double Stranded RNA Structures and Constructs and Methods for Generating and Using the Same," filed 31 Jul 2003; as well as the "udderly" structured hairpins, hairpins with mismatched regions, and multiepitope constructs as taught in USSN 60/419,532, filed 18 Oct 2002, and PCT/US2003/033466, "Double Stranded RNA Structures and Constructs, and Methods for Generating and Using the Same," filed 20 Oct 2003.

By "short dsRNA" is meant a dsRNA that has about 50, 45, 40, 35, 30, 27, 25, 23, 21, 20 or 19 contiguous nucleotides in length that are in a double stranded conformation. Desirably, the short dsRNA is at least 19 basepairs in length. In desirable embodiments, the double stranded region is between 19 to 50, 19 to 40, 19 to 30, 19 to 25, 20 to 25, 21 to 23, 25 to 30, or 30 to 40 contiguous basepairs in length, inclusive. In some embodiments, the short dsRNA is between 30 to 50, 50 to 100, 100 to 200, 200 to 300, 400 to 500, 500 to 700, 700 to 1000, 1000 to 2000, or 2000 to 5000 nucleotides in length, inclusive and has a double stranded region that is between 38 and 60 contiguous basepairs in length, inclusive. In one embodiment, the short dsRNA is completely double stranded. In some embodiments, the short dsRNA is between 11 and 30 nucleotides in length, and the entire dsRNA is double stranded. In other embodiments, the short dsRNA has one or two single stranded regions. In particular embodiments, the short dsRNA binds PKR or another protein in a dsRNA mediated stress response pathway. Desirably, the short dsRNA inhibits the dimerization and activation of PKR by at least 20, 40, 60, 80, 90, or

100%. In some desirable embodiments, the short dsRNA inhibits the binding of a long dsRNA to PKR or another component of a dsRNA mediated stress response pathway by at least 20, 40, 60, 80, 90, or 100%. See also the teaching of USSN 10/425,006, filed 28 Apr 2003, "Methods of Silencing Genes Without Inducing Toxicity", Pachuk, as to utilization of short dsRNAs in conjunction with other dsRNAs to avoid dsRNA mediated toxicity.

5 By "at least 19 contiguous base pair nucleotide sequence" is meant that a nucleotide sequence can start at any nucleotide within one of the disclosed sequences, so long as the start site is capable of producing a polynucleotide of at least 19 base pairs. For example, an at least 10 19 contiguous base pair nucleotide sequence can comprise nucleotide 1 through nucleotide 19, nucleotide 2 through nucleotide 20, nucleotide 3 through nucleotide 21, and so forth to produce a 19mer. Thus, a 20mer can comprise nucleotide 1 through nucleotide 20, nucleotide 2 through nucleotide 21, nucleotide 3 through nucleotide 22, and so forth. Similar sequences above 20 contiguous nucleotides are envisioned.

15 By "dsRNA" or "dsRNA effector molecule" is meant a nucleic acid containing a region of two or more nucleotides that are in a double stranded conformation. It is envisioned that the conserved viral sequences of the invention may be utilized in any of the many compositions of "dsRNA effector molecules" known in the art or subsequently developed which act through a dsRNA-mediated gene silencing or RNAi mechanism, including, e.g., "hairpin" or stem-loop double-stranded RNA effector molecules in which a single RNA strand with self-complementary 20 sequences is capable of assuming a double-stranded conformation, or duplex dsRNA effector molecules comprising two separate strands of RNA,. In various embodiments, the dsRNA consists entirely of ribonucleotides or consists of a mixture of ribonucleotides and deoxynucleotides, such as the RNA/DNA hybrids disclosed, for example, by WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. The dsRNA or dsRNA effector 25 molecule may be a single molecule with a region of self-complementarity such that nucleotides in one segment of the molecule base pair with nucleotides in another segment of the molecule. In various embodiments, a dsRNA that consists of a single molecule consists entirely of 30 ribonucleotides or includes a region of ribonucleotides that is complementary to a region of deoxyribonucleotides. Alternatively, the dsRNA may include two different strands that have a region of complementarity to each other. In various embodiments, both strands consist entirely of ribonucleotides, one strand consists entirely of ribonucleotides and one strand consists entirely of deoxyribonucleotides.

of deoxyribonucleotides, or one or both strands contain a mixture of ribonucleotides and deoxyribonucleotides. Desirably, the regions of complementarity are at least 70, 80, 90, 95, 98, or 100% complementary to each other and to a target nucleic acid sequence. Desirably, the region of the dsRNA that is present in a double stranded conformation includes at least 19, 20,

5 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 50, 75, 100, 200, 500, 1000, 2000 or 5000 nucleotides or includes all of the nucleotides in a cDNA or other target nucleic acid sequence being represented in the dsRNA. In some embodiments, the dsRNA does not contain any single stranded regions, such as single stranded ends, or the dsRNA is a hairpin. In other embodiments, the dsRNA has one or more single stranded regions or overhangs. Desirable RNA/DNA hybrids include a DNA

10 strand or region that is an antisense strand or region (e.g. has at least 70, 80, 90, 95, 98, or 100% complementarity to a target nucleic acid) and an RNA strand or region that is a sense strand or region (e.g. has at least 70, 80, 90, 95, 98, or 100% identity to a target nucleic acid), and vice versa. In various embodiments, the RNA/DNA hybrid is made in vitro using enzymatic or chemical synthetic methods such as those described herein or those described in WO 00/63364,

15 filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. In other embodiments, a DNA strand synthesized in vitro is complexed with an RNA strand made in vivo or in vitro before, after, or concurrent with the transformation of the DNA strand into the cell. In yet other embodiments, the dsRNA is a single circular nucleic acid containing a sense and an antisense region, or the dsRNA includes a circular nucleic acid and either a second circular nucleic acid or

20 a linear nucleic acid (see, for example, WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999.) Exemplary circular nucleic acids include lariat structures in which the free 5' phosphoryl group of a nucleotide becomes linked to the 2' hydroxyl group of another nucleotide in a loop back fashion.

In other embodiments, the dsRNA includes one or more modified nucleotides in which

25 the 2' position in the sugar contains a halogen (such as fluorine group) or contains an alkoxy group (such as a methoxy group) which increases the half-life of the dsRNA in vitro or in vivo compared to the corresponding dsRNA in which the corresponding 2' position contains a hydrogen or an hydroxyl group. In yet other embodiments, the dsRNA includes one or more linkages between adjacent nucleotides other than a naturally-occurring phosphodiester linkage.

30 Examples of such linkages include phosphoramidate, phosphorothioate, and phosphorodithioate linkages. The dsRNAs may also be chemically modified nucleic acid molecules as taught in

U.S. Patent No. 6,673,661. In other embodiments, the dsRNA contains one or two capped strands, as disclosed, for example, by WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. In other embodiments, the dsRNA contains coding sequence or non-coding sequence, for example, a regulatory sequence (e.g., a transcription factor binding 5 site, a promoter, or a 5' or 3' untranslated region (UTR) of an mRNA). Additionally, the dsRNA can be any of the at least partially dsRNA molecules disclosed in WO 00/63364, filed April 19, 2000 (see, for example, pages 8-22), as well as any of the dsRNA molecules described in US Provisional Application 60/399,998 filed July 31, 2002, and PCT/US2003/024028, filed 31-Jul-2003; and US Provisional Application 60/419,532 filed October 18, 2002, and 10 PCT/US2003/033466, filed 20-Oct-2003, the teaching of which is hereby incorporated by reference. Any of the dsRNAs may be expressed in vitro or in vivo using the methods described herein or standard methods, such as those described in WO 00/63364, filed April 19, 2000 (see, for example, pages 16-22). In some preferred embodiments, multiple anti-HBV and/or anti-HCV dsRNA effector molecules of the invention are transcribed in a mammalian cell from one 15 or more expression constructs each comprising multiple polymerase III promoter expression cassettes as described in more detail in US 60/603622; US 60/629942; and PCT/US05/29976 filed 23-August-2005; "Multiple Polymerase III Promoter Expression Constructs"; the teaching of which is incorporated by reference.

dsRNA "Hairpin" Constructs or dsRNA "Hairpin" Expression Vectors: Constructs 20 encoding a unimolecular hairpin dsRNA are more desirable for some applications than constructs encoding duplex dsRNA (i.e., dsRNA composed of one RNA molecule with a sense region and a separate RNA molecule with an antisense region) because the single-stranded RNA with inverted repeat sequences more efficiently forms a dsRNA hairpin structure. This greater efficiency is due in part to the occurrence of transcriptional interference arising in vectors containing 25 converging promoters that generate duplex dsRNA. Transcriptional interference results in the incomplete synthesis of each RNA strand thereby reducing the number of complete sense and antisense strands that can base-pair with each other and form duplexes. Transcriptional interference can be overcome, if desired, through the use of (i) a two vector system in which one vector encodes the sense RNA and the second vector encodes the antisense RNA, (ii) a bicistronic vector in which the individual strands are encoded by the same plasmid but through 30 the use of separate cistrons, or (iii) a single promoter vector that encodes a hairpin dsRNA, i.e.,

an RNA in which the sense and antisense sequences are encoded within the same RNA molecule. Hairpin-expressing vectors have some advantages relative to the duplex vectors. For example, in vectors that encode a duplex RNA, the RNA strands need to find and base-pair with their complementary counterparts soon after transcription. If this hybridization does not happen, the 5 individual RNA strands diffuse away from the transcription template and the local concentration of sense strands with respect to antisense strands is decreased. This effect is greater for RNA that is transcribed intracellularly compared to RNA transcribed in vitro due to the lower levels of template per cell. Moreover, RNA folds by nearest neighbor rules, resulting in RNA molecules that are folded co-transcriptionally (i.e., folded as they are transcribed). Some percentage of 10 completed RNA transcripts is therefore unavailable for base-pairing with a complementary second RNA because of intra-molecular base-pairing in these molecules. The percentage of such unavailable molecules increases with time following their transcription. These molecules may never form a duplex because they are already in a stably folded structure. In a hairpin RNA, an 15 RNA sequence is always in close physical proximity to its complementary RNA. Since RNA structure is not static, as the RNA transiently unfolds, its complementary sequence is immediately available and can participate in base-pairing because it is so close. Once formed, the hairpin structure is predicted to be more stable than the original non-hairpin structure. Especially desirable are, e.g., "forced" hairpin constructs, partial hairpins capable of being 20 extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002; and PCT/US2003/024028, "Double Stranded RNA Structures and Constructs and Methods for Generating and Using the Same," filed 31-Jul-2003; as well as the "udderly" structured hairpins, hairpins with mismatched regions, and multiepitope constructs 25 as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, "Double-Stranded RNA Structures and Constructs, and Methods for Generating and Using the Same," filed 20-Oct-2003.

By "short dsRNA" is meant a dsRNA that has about 200, 100, 75, 50, 45, 40, 35, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20 or 19 contiguous nucleotides in length that are in a double stranded conformation. Desirably, the short dsRNA comprises a double-stranded region of at least 19 contiguous basepairs in length identical/complementary to a target sequence to be 30 inhibited. In desirable embodiments, the double stranded region is between 19 to 50, 19 to 40, 19 to 30, 19 to 25, 20 to 25, 21 to 23, 25 to 30, or 30 to 40 contiguous basepairs in length,

inclusive. In some embodiments, the short dsRNA is between 30 to 50, 50 to 100, 100 to 200, 200 to 300, 400 to 500, 500 to 700, 700 to 1000, 1000 to 2000, or 2000 to 5000 nucleotides in length, inclusive and has a double stranded region that is between 38 and 60 contiguous basepairs in length, inclusive. In one embodiment, the short dsRNA is completely double stranded. In some embodiments, the short dsRNA is between 11 and 30 nucleotides in length, and the entire dsRNA is double stranded. In other embodiments, the short dsRNA has one or two single stranded regions. In some embodiments, the short dsRNA is a “shRNA” or “short-hairpin RNA” or “shRNA effector molecule” or “dsRNA hairpin”, meaning an RNA molecule of less than approximately 400 to 500 nucleotides (nt) in length, preferably less than 100 to 200 nt in length, in which at least one stretch of at least about 15 to 100 nucleotides (preferably 17 to 50 nt; more preferably 19 to 29 nt) is base paired with a complementary sequence located on the same RNA molecule, and where said sequence and complementary sequence are separated by an unpaired region of at least about 4 to 7 nucleotides (preferably about 9 to about 15 nucleotides) which forms a single-stranded loop above the stem structure created by the two regions of base complementarity. The shRNA molecules comprise at least one stem-loop structure comprising a double-stranded stem region of about 17 to about 100 bp; about 17 to about 50 bp; about 40 to about 100 bp; about 18 to about 40 bp; or from about 19 to about 29 bp; homologous and complementary to a target sequence to be inhibited; and an unpaired loop region of at least about 4 to 7 nucleotides; preferably about 9 to about 15 nucleotides, which forms a single-stranded loop above the stem structure created by the two regions of base complementarity. Included shRNAs are dual or bi-finger (i.e., having two stem-loop structures) and multi-finger hairpin dsRNAs (having multiple stem-loop structures), in which the RNA molecule comprises two or more of such stem-loop structures separated by single-stranded spacer regions. In some embodiments, an expression construct may be used to express one or more of such shRNA molecules in a mammalian cell, including multiple copies of the same, and/or one or more, including multiple different, short hairpin RNA molecules. Short hairpin RNA molecules considered to be the “same” as each other are those that comprise only the same double-stranded sequence, and short hairpin RNA molecules considered to be “different” from each other will comprise different double-stranded sequences, regardless of whether the sequences to be targeted by each different double-stranded sequence are within the same, or a different gene, such as, e.g.,

sequences of a promoter region and of a transcribed region (mRNA) of the same gene, or sequences of two different genes.

In particular embodiments, the short dsRNA binds PKR or another protein in a dsRNA-mediated stress response pathway. Desirably, such a short dsRNA inhibits the dimerization and activation of PKR by at least 20, 40, 60, 80, 90, or 100%. In some desirable embodiments, the short dsRNA inhibits the binding of a long dsRNA to PKR or another component of a dsRNA-mediated stress response pathway by at least 20, 40, 60, 80, 90, or 100%. See also the teaching of USSN 10/425,006, filed 28-Apr-2003, “Methods of Silencing Genes Without Inducing Toxicity”, Pachuk, as to utilization of short dsRNAs in conjunction with other dsRNAs to avoid dsRNA-mediated toxicity. The applicants have demonstrated, however, that dsRNA molecules, even long dsRNA molecules, are in general unlikely to evoke a significant dsRNA stress response, including a PKR or interferon or “panic” response, if they are expressed intracellularly in the mammalian (or other vertebrate) cell in which the RNAi effect is desired. See, e.g., US 2002/0132257, “Use of post-transcriptional gene silencing for identifying nucleic acid sequences that modulate the function of a cell”. Accordingly, such “expressed interfering RNA molecules” or “eiRNA” molecules and “eiRNA expression constructs”, i.e., dsRNA molecules (or the corresponding dsRNA expression constructs) expressed intracellularly or endogenously in vivo within the mammalian cell in which dsRNA gene silencing or RNAi is induced, are preferred in some aspects of the invention.

By “at least 19 contiguous base pair nucleotide sequence” is meant that a nucleotide sequence can start at any nucleotide within one of the disclosed sequences, so long as the start site is capable of producing a polynucleotide of at least 19 contiguous base pairs. For example, an at least 19 contiguous base pair nucleotide sequence can comprise nucleotide 1 through nucleotide 19, nucleotide 2 through nucleotide 20, nucleotide 3 through nucleotide 21, and so forth to produce a 19mer. Thus, a 20mer can comprise nucleotide 1 through nucleotide 20, nucleotide 2 through nucleotide 21, nucleotide 3 through nucleotide 22, and so forth. Similar sequences above 20 contiguous nucleotides, e.g., 21, 22, 23, 24, 25, 26, 27, etc. selected from within the conserved sequences are envisioned. Such a sequence of at least 19 contiguous nucleotides (in double-stranded conformation with its complement) is “an at least 19 contiguous base pair sequence” and may be present as a duplex dsRNA, within a dsRNA hairpin, or encoded in a dsRNA expression construct.

By "expression vector" is meant any double stranded DNA or double stranded RNA designed to transcribe an RNA, e.g., a construct that contains at least one promoter operably linked to a downstream gene or coding region of interest (e.g., a cDNA or genomic DNA fragment that encodes a protein, or any RNA of interest, optionally, e.g., operatively linked to sequence lying outside a coding region, an antisense RNA coding region, a dsRNA coding region, or RNA sequences lying outside a coding region). Transfection or transformation of the expression vector into a recipient cell allows the cell to express RNA or protein encoded by the expression vector. An expression vector may be a genetically engineered plasmid, virus, or artificial chromosome derived from, for example, a bacteriophage, adenovirus, retrovirus, poxvirus, or herpesvirus.

By an "expression construct" is meant any double stranded DNA or double stranded RNA designed to transcribe an RNA, e.g., a construct that contains at least one promoter operably linked to a downstream gene or coding region of interest (e.g., a cDNA or genomic DNA fragment that encodes a protein, or any RNA of interest). Transfection or transformation of the expression construct into a recipient cell allows the cell to express RNA or protein encoded by the expression construct. An expression construct may be a genetically engineered plasmid, virus, or artificial chromosome derived from, for example, a bacteriophage, adenovirus, retrovirus, poxvirus, or herpesvirus. An expression construct does not have to be replicable in a living cell, but may be made synthetically.

By "operably linked" is meant that a nucleic acid molecule and one or more regulatory sequences (e.g., a promoter) are connected in such a way as to permit transcription of the mRNA or permit expression and/or secretion of the product (i.e., a polypeptide) of the nucleic acid molecule when the appropriate molecules are bound to the regulatory sequences.

By a "promoter" is meant a nucleic acid sequence sufficient to direct transcription of a covalently linked nucleic acid molecule. Also included in this definition are those transcription control elements (e.g., enhancers) that are sufficient to render promoter dependent gene expression controllable in a cell type specific, tissue specific, or temporal specific manner, or that are inducible by external signals or agents; such elements, which are well known to skilled artisans, may be found in a 5' or 3' region of a gene or within an intron. Desirably a promoter is operably linked to a nucleic acid sequence, for example, a cDNA or a gene in such a way as to permit expression of the nucleic acid sequence.

The RNA molecule according to this invention may be delivered to the mammalian or extracellular pathogen present in the mammalian cell in the composition as an RNA molecule or partially double stranded RNA sequence, or RNA/DNA hybrid, which was made *in vitro* by conventional enzymatic synthetic methods using, for example, the bacteriophage T7, T3 or SP6 RNA polymerases according to the conventional methods described by such texts as the Promega Protocols and Applications Guide, (3rd ed. 1996), eds. Doyle, ISBN No. 1-57 Alternately these molecules may be made by chemical synthetic methods *in vitro* [see, e.g., Q. Xu et al., Nucleic Acids Res., 24(18):3643-4 (Sept. 1996); N. Naryshkin et al., Bioorg. Khim., 22(9):691-8 (Sept. 1996); J. A. Grasby et al., Nucleic Acids Res., 21(19):4444-50 (Sept. 1993); C. Chaix et al., Nucleic Acids Res. 17:7381-93 (1989); S.H. Chou et al., Biochem., 28(6):2422-35 (Mar. 1989); O. Odal et al., Nucleic Acids Symp. Ser., 21:105-6 (1989); N.A. Naryshkin et al., Bioorg. Khim, 22(9):691-8 (Sept. 1996); S. Sun et al., RNA, 3(11):1352-1363 (Nov. 1997); X. Zhang et al., Nucleic Acids Res., 25(20):3980-3 (Oct. 1997); S. M. Grvaznov et al., Nucleic Acids Res., 26(18):4160-7 (Sept. 1998); M. Kadokura et al., Nucleic Acids Symp. Ser., 37:77-8 (1997); A. Davison et al., Biomed. Pept. Proteins. Nucleic Acids, 2(I):1-6 (1996); and A. V. Mudrakovskaia et al., Bioorg. Khim., 17(6):819-22 (Jun. 1991)].

By an "expression construct" is meant any double-stranded DNA or double-stranded RNA designed to transcribe an RNA, e.g., a construct that contains at least one promoter operably linked to a downstream gene or coding region of interest (e.g., a cDNA or genomic DNA fragment that encodes a protein, or any RNA of interest). Transfection or transformation of the expression construct into a recipient cell allows the cell to express RNA or protein encoded by the expression construct. An expression construct may be a genetically engineered plasmid, virus, or artificial chromosome derived from, for example, a bacteriophage, adenovirus, retrovirus, poxvirus, or herpesvirus. An expression construct does not have to be replicable in a living cell, but may be made synthetically. An expression construct or expression vector engineered to express a double-stranded RNA effector molecule or dsRNA molecule is a "dsRNA expression construct" or "dsRNA expression vector".

In one embodiment of the invention, a recombinant expression vector or expression construct is engineered to express multiple, e.g., three, four, five or more short hairpin dsRNA effector molecules, each expressed from a different expression cassette comprising a polymerase III promoter, one or more, including all of which, may be different from the others. In one

aspect of the invention, a recombinant expression vector transcribing three, four, five or more different shRNA molecules (each comprising a double-stranded “stem” region comprising at least 19 contiguous basepairs from/complementary to a conserved HBV and/or HCV sequence) is used to inhibit replication of hepatitis B virus (HBV) and/or hepatitis C virus (HCV). In one embodiment, each shRNA molecule is expressed under the control of a polymerase III promoter, e.g., 7SK, H1, and U6, which may be the same or different. Such dsRNA expression constructs comprising multiple polymerase III expression cassettes are described in greater detail in PCT/US05/29976, “Multiple Polymerase III Promoter Expression Constructs”, the teaching of which is hereby incorporated by reference. In one aspect, a recombinant expression vector or expression construct of the invention may express one or more bi-fingered or multi-fingered dsRNA hairpin molecules from one or more polymerase III promoter-driven transcription units as well as one or more single hairpin dsRNA molecules from one or more polymerase III promoter-driven transcription units. It will be understood that in any of said expression constructs transcribing a hairpin dsRNA from a polymerase III promoter, the hairpin dsRNA may be a single hairpin dsRNA or a bi-fingered, or multi-fingered dsRNA hairpin as described in WO2004/035765, published 29-Apr-2004, or a partial or forced hairpin structure as described in WO2004/011624, published 5-Feb-2004, the teaching of which is incorporated herein by reference.

By “operably linked” is meant that a nucleic acid sequence or molecule and one or more regulatory sequences (e.g., a promoter, enhancer, repressor, terminator) are connected in such a way as to permit transcription of an RNA molecule, e.g., a single-stranded RNA molecule such as a sense, antisense, a dsRNA hairpin, or an mRNA, or permit expression and translation and/or secretion of the product (i.e., a polypeptide) of the nucleic acid molecule when the appropriate molecules are bound to the regulatory sequences.

By a “promoter” is meant a nucleic acid sequence sufficient to direct transcription of a covalently linked nucleic acid molecule. Also included in this definition are those transcription control elements (e.g., enhancers) that are sufficient to render promoter-dependent gene expression controllable in a cell type-specific, tissue-specific, or temporal-specific manner, or that are inducible by external signals or agents; such elements, which are well-known to skilled artisans, may be found in a 5’ or 3’ region of a gene or within an intron. See, e.g., published U.S. Patent Application No. 2005/0130184 A1, 16-June-2005, Xu et al., directed to modified

polymerase III promoters which utilize polymerase II enhancer elements, as well as Published U.S. Patent Application No. 2005/0130919 A1, 16-June-2005, Xu et al., directed to regulatable polymerase III and polymerase II promoters, the teaching of which is hereby incorporated by reference. Desirably a promoter is operably linked to a nucleic acid sequence, for example, a
 5 cDNA or a gene sequence, or a sequence encoding a dsRNA, e.g., a shRNA, in such a way as to permit expression of the nucleic acid sequence.

The RNA molecule according to this invention may be delivered to the mammalian cell or extracellular pathogen present in the mammalian cell in the composition as a dsRNA effector molecule or partially double stranded RNA sequence, or RNA/DNA hybrid, which was made in
 10 vitro by conventional enzymatic synthetic methods using, for example, the bacteriophage T7, T3 or SP6 RNA polymerases according to the conventional methods described by such texts as the Promega Protocols and Applications Guide, (3rd ed. 1996), eds. Doyle, ISBN No. 1 57
 Alternatively these molecules may be made by chemical synthetic methods in vitro [see, e.g., Q.
 15 Xu et al., Nucleic Acids Res., 24(18):3643-4 (Sept. 1996); N. Naryshkin et al., Bioorg. Khim., 22(9):691-8 (Sept. 1996); J. A. Grasby et al., Nucleic Acids Res., 21(19):4444-50 (Sept 1993);
 C. Chaix et al., Nucleic Acids Res. 17:7381-93 (1989); S.H. Chou et al., Biochem., 28(6):2422-35 (Mar. 1989); O. Odal el al., Nucleic Acids Symp. Ser., 21:105-6 (1989); N.A. Naryshkin et al.,
 Bioorg. Khim, 22(9):691-8 (Sept. 1996); S. Sun et al., RNA, 3(11):1352-1363 (Nov. 1997); X.
 Zhang et al., Nucleic Acids Res., 25(20):3980-3 (Oct. 1997); S. M. Grvaznov el al., Nucleic
 20 Acids Res., 2-6 (18):4160-7 (Sept. 1998); M. Kadokura et al., Nucleic Acids Symp. Ser., 37:77-8 (1997); A. Davison et al., Biomed. Pept. Proteins. Nucleic Acids, 2(I):1-6 (1996); and A. V.
 Mudrakovskaia et al., Bioorg. Khirn., 17(6):819-22 (Jun. 1991)].

Still alternatively, the RNA molecule of this invention can be made in a recombinant microorganism, e.g., bacteria and yeast or in a recombinant host cell, e.g., mammalian cells, and
 25 isolated from the cultures thereof by conventional techniques. See, e.g., the techniques described in Sambrook et al, MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, which is exemplary of laboratory manuals that detail these techniques, and the techniques described in US Patent Nos. 5,824,538; 5,877,159; and 5,643,771, incorporated herein by reference.

30 Such RNA molecules prepared or synthesized *in vitro* may be directly delivered to the mammalian cell or to the mammal as they are made *in vitro*. The references above provide one

of skill in the art with the techniques necessary to produce any of the following specific embodiments, given the teachings provided herein. Therefore, in one embodiment, the "agent" of the composition is a duplex (i.e., it is made up of two strands), either complete or partially double stranded RNA.

5 In another embodiment, the agent is a single stranded RNA sense strand. In another embodiment, the agent of the composition is a single stranded RNA anti-sense strand.

~~Preferably the single stranded RNA sense or anti sense strand forms a hairpin at one or both termini. Desirably, the single stranded RNA sense or anti sense strand forms a hairpin at some intermediate portion between the termini. Such a single stranded RNA sense or anti sense strand may also be designed to fold back upon itself to become partially double stranded *in vitro* or *in vivo*. Yet another embodiment of an extant RNA molecule as the effective agent used in the compositions is a single stranded RNA sequence comprising both a sense polynucleotide sequence and an antisense polynucleotide sequence, optionally separated by a non-base paired polynucleotide sequence. Preferably, this single stranded RNA sequence has the ability to become double stranded once it is in the cell, or *in vitro* during its synthesis.~~

Preferably the single stranded RNA sense or anti-sense strand forms a hairpin at one or both termini. Desirably, the single stranded RNA sense or anti- sense strand forms a hairpin at some intermediate portion between the termini. Such a single stranded RNA sense or anti-sense strand may also be designed to fold back upon itself to become partially double stranded *in vitro* or *in vivo*. Yet another embodiment of an extant RNA molecule as the effective agent used in the compositions is a single stranded RNA sequence comprising both a sense polynucleotide sequence and an antisense polynucleotide sequence, optionally separated by a non-base paired polynucleotide sequence. Preferably, this single stranded RNA sequence has the ability to become double-stranded once it is in the cell, or *in vitro* during its synthesis. In desirable embodiments, a sequence of at least about 19 to 29 contiguous basepairs will assume a double-stranded conformation. In desirable embodiments, the double-stranded region will include an at least about 19 contiguous basepair sequence identical/complementary to a target nucleotide sequence to be downregulated or inhibited.

Still another embodiment of this invention is an RNA/DNA hybrid as described above.

30 Still another embodiment of the synthetic RNA molecule is a circular RNA molecule that optionally forms a rod structure [see, e.g., K-S. Wang et al., Nature 323:508-514 (1986)] or is

partially double-stranded, and can be prepared according to the techniques described in S. Wang et al., Nucleic Acids Res., 22(12):2326-33 (June 1994); Y. Matsumoto et al., Proc. Natl. Acad. Sci., USA, 87(19):7628-32 (Oct. 1990); E. Ford & M. Ares, Proc. Natl. Acad. Sci. USA 91(8):3117-21 (Apr. 1994); M. Tsagris et al., Nucleic Acids Res., 19 7):1605-12 (Apr. 1991); S.

5 Braun et al., Nucleic Acids Res. 24(21):4152-7 (Nov. 1996); Z. Pasman et al., RNA, 2(6):603-10 (Jun. 1996); P. G. Zaphiropoulos, Proc. Natl. Acad. Sci., USA, 93(13):6536-41 (Jun. 1996); D. Beaudry et al., Nucleic Acids Res., 23(15):3064-6 (Aug. 1995), all incorporated herein by reference. Still another agent is a double-stranded molecule comprised of RNA and DNA present on separate strands, or interspersed on the same strand.

10 ~~Alternatively, the RNA molecule may be formed *in vivo* and thus delivered by a "delivery agent" which generates such a partially double stranded RNA molecule *in vivo* after delivery of the agent to the mammalian cell or to the mammal. Thus, the agent which forms the composition of this invention is, in one embodiment, a double stranded DNA molecule "encoding" one of the above described RNA molecules. The DNA agent provides the nucleotide sequence which is transcribed within the cell to become a double stranded RNA. In another embodiment, the DNA sequence provides a deoxyribonucleotide sequence which within the cell is transcribed into the above described single stranded RNA sense or anti sense strand, which optionally forms a hairpin at one or both termini or folds back upon itself to become partially double stranded. The DNA molecule which is the delivery agent of the composition can provide a single stranded RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide sequence, optionally separated by a nonbase paired polynucleotide sequence, and wherein the single stranded RNA sequence has the ability to become double stranded. Alternatively, the DNA molecule which is the delivery agent provides for the transcription of the above described circular RNA molecule that optionally forms a rod structure or partial double strand *in vivo*. The DNA molecule may also provide for the *in vivo* production of an RNA/DNA hybrid as described above, or a duplex containing one RNA strand and one DNA strand. These various DNA molecules may be designed by resort to conventional techniques such as those described in Sambrook, cited above or in the Promega reference, cited above.~~

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~~A latter delivery agent of the present invention, which enables the formation in the mammalian cell of any of the above described RNA molecules, can be a DNA single stranded or~~

double stranded plasmid or vector. Expression vectors designed to produce RNAs as described herein *in vitro* or *in vivo* may contain sequences under the control of any RNA polymerase, including mitochondrial RNA polymerase, RNA pol I, RNA pol II, and RNA pol III, and viral polymerases, and bacteriophage polymerases such as T7 and Sp6. These vectors can be used to transcribe the desired RNA molecule in the cell according to this invention. Vectors may be desirably designed to utilize an endogenous mitochondrial RNA polymerase (e.g., human mitochondrial RNA polymerase, in which case such vectors may utilize the corresponding human mitochondrial promoter). Mitochondrial polymerases may be used to generate capped (through expression of a capping enzyme) or uncapped messages *in vivo*. RNA pol I, RNA pol II, and RNA pol III transcripts may also be generated *in vivo*. Such RNAs may be capped or not, and if desired, cytoplasmic capping may be accomplished by various means including use of a capping enzyme such as a vaccinia capping enzyme or an alphavirus capping enzyme. However, all pol II transcripts are capped. The DNA vector is designed to contain one of the promoters or multiple promoters in combination (mitochondrial, RNA pol I, pol II, or pol III, or viral, bacterial or bacteriophage promoters along with the cognate polymerases). Preferably, where the promoter is RNA pol II, the sequence encoding the RNA molecule has an open reading frame greater than about 300 nts and must follow the rules of design to prevent nonsense mediated degradation in the nucleus. Such plasmids or vectors can include plasmid sequences from bacteria, viruses or phages.

Such vectors include chromosomal, episomal and virus derived vectors e.g., vectors derived from bacterial plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses, vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids and phagemids.

Alternatively, the RNA molecule may be formed *in vivo* and thus delivered by a “delivery agent” which generates such a partially double-stranded RNA molecule *in vivo* after delivery of the agent to the mammalian cell or to the mammal. Thus, the agent which forms the composition of this invention is, in one embodiment, a double stranded DNA molecule “encoding” one of the above-described RNA molecules, e.g., a dsRNA expression vector or expression construct. The DNA agent provides the nucleotide sequence which is transcribed within the cell to become a double stranded RNA. In another embodiment, the DNA sequence provides a deoxyribonucleotide sequence which within the cell is transcribed into the above-

described single stranded RNA sense or anti-sense strand, which optionally forms a hairpin at one or both termini or folds back upon itself to become partially double stranded. The DNA molecule which is the delivery agent of the composition can provide a single stranded RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide sequence, optionally separated by a nonbase paired polynucleotide sequence, and wherein the single stranded RNA sequence has the ability to become double-stranded. Alternatively, the DNA molecule which is the delivery agent provides for the transcription of the above-described circular RNA molecule that optionally forms a rod structure or partial double strand in vivo. The DNA molecule may also provide for the in vivo production of an RNA/DNA hybrid as described above, or a duplex containing one RNA strand and one DNA strand. These various DNA molecules may be designed by resort to conventional techniques such as those described in Sambrook, cited above or in the Promega reference, cited above.

A latter delivery agent of the present invention, which enables the formation in the mammalian cell of any of the above-described RNA molecules, can be a DNA single stranded or double stranded plasmid or vector. Expression vectors designed to produce RNAs as described herein in vitro or in vivo may contain sequences under the control of any RNA polymerase, including mitochondrial RNA polymerase, RNA pol I, RNA pol II, and RNA pol III, and viral polymerases, and bacteriophage polymerases such as T7 and Sp6. Desirably, expression vectors designed for in vivo expression of dsRNA effector molecules within a mammalian cell may be designed to utilize an endogenous mammalian polymerase such as an RNA polymerase I, RNA polymerase II, RNA polymerase III, and/or a mitochondrial polymerase. Expression vectors utilizing cognate promoter(s), e.g., polymerase III promoters such as U6, H1, or 7SK, in order to effect transcription by RNA polymerase III can readily be designed. Preferred for expression of short RNA molecules less than about 400 to 500 nucleotides in length are RNA polymerase III promoters. In some aspects, an "RNA polymerase III promoter" or "RNA pol III promoter" or "polymerase III promoter" or "pol III promoter" is preferred, meaning any invertebrate, vertebrate, or mammalian promoter, e.g., human, murine, porcine, bovine, primate, simian, etc. that, in its native context in a cell, associates or interacts with RNA polymerase III to transcribe its operably linked gene, or any variant thereof, natural or engineered, that will interact in a selected host cell with an RNA polymerase III to transcribe an operably linked nucleic acid sequence. Preferred in some applications are the Type III RNA pol III promoters including U6,

H1, 7SK, and MRP which exist in the 5' flanking region, include TATA boxes, and lack internal promoter sequences. One reason RNA Pol III promoters are especially desirable for expression of small engineered RNA transcripts is that RNA Pol III termination, unlike RNA polymerase II termination, occurs efficiently and precisely at a short run of thymine residues in the DNA

5 coding strand, without other protein factors, T4 and T5 being the shortest Pol III termination signals in yeast and mammals, with oligo (dT) terminators longer than T5 being very rare in mammals. Accordingly, the multiple polymerase III promoter expression constructs of the invention will include an appropriate oligo (dT) termination signal, i.e., a sequence of 4, 5, 6 or more Ts, operably linked 3' to each RNA Pol III promoter in the DNA coding strand.

10 These vectors can be used to transcribe the desired RNA molecule in the cell according to this invention. Vectors may be desirably designed to utilize an endogenous mitochondrial RNA polymerase (e.g., human mitochondrial RNA polymerase, in which case such vectors may utilize the corresponding human mitochondrial promoter). Mitochondrial polymerases may be used to generate capped (through expression of a capping enzyme) or uncapped messages in vivo. RNA

15 pol I, RNA pol II, and RNA pol III transcripts may also be generated in vivo. Such RNAs may be capped or not, and if desired, cytoplasmic capping may be accomplished by various means including use of a capping enzyme such as a vaccinia capping enzyme or an alphavirus capping enzyme. However, all pol II transcripts are capped. The DNA vector is designed to contain one of the promoters or multiple promoters in combination (mitochondrial, RNA pol I, pol II, or pol III, or viral, bacterial or bacteriophage promoters along with the cognate polymerases).

20 Preferably, where the promoter is RNA pol II, the sequence encoding the RNA molecule has an open reading frame greater than about 300 nts and must follow the rules of design to prevent nonsense-mediated degradation in the nucleus. Such plasmids or vectors can include plasmid sequences from bacteria, viruses or phages.

25 Such vectors include chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses, vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids and phagemids.

30 Thus, one exemplary vector is a single or double-stranded phage vector. Another exemplary vector is a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for

introducing DNA and RNA into cells. The vectors, in the case of phage and viral vectors may also be and preferably are introduced into cells as packaged or encapsidated virus by well known techniques for infection and transduction. Viral vectors may be replication competent or replication defective. In the latter case, viral propagation generally occurs only in
5 complementing host cells.

In another embodiment the delivery agent comprises more than a single DNA or RNA plasmid or vector. As one example, a first DNA plasmid can provide a single stranded RNA sense polynucleotide sequence as described above, and a second DNA plasmid can provide a single stranded RNA anti-sense polynucleotide sequence as described above, wherein the sense
10 and anti-sense RNA sequences have the ability to base-pair and become double-stranded. Such plasmid(s) can comprise other conventional plasmid sequences, e.g., bacterial sequences such as the well-known sequences used to construct plasmids and vectors for recombinant expression of a protein. However, it is desirable that the sequences which enable protein expression, e.g., Kozak regions, etc., are not included in these plasmid structures.

15 The vectors designed to produce dsRNAs of the invention may desirably be designed to generate two or more, including a number of different dsRNAs homologous and complementary to a target sequence. This approach is desirable in that a single vector may produce many, independently operative dsRNAs rather than a single dsRNA molecule from a single transcription unit and by producing a multiplicity of different dsRNAs, it is possible to self select
20 for optimum effectiveness. Various means may be employed to achieve this, including autocatalytic sequences as well as sequences for cleavage to create random and/or predetermined splice sites.

Other delivery agents for providing the information necessary for formation of the above-described desired RNA molecules in the mammalian cell include live, attenuated or killed,
25 inactivated recombinant bacteria which are designed to contain the sequences necessary for the required RNA molecules of this invention. Such recombinant bacterial cells, fungal cells and the like can be prepared by using conventional techniques such as described in US Patent Nos. 5,824,538; 5,877,159; and 5,643,771, incorporated herein by reference. Microorganisms useful in preparing these delivery agents include those listed in the above cited reference,
30 including, without limitation, Escherichia coli, Bacillus subtilis, Salmonella typhimurium, and various species of Pseudomonas, Streptomyces, and Staphylococcus.

Still other delivery agents for providing the information necessary for formation of the desired, above-described RNA molecules in the mammalian cell include live, attenuated or killed, inactivated viruses, and particularly recombinant viruses carrying the required RNA polynucleotide sequence discussed above. Such viruses may be designed similarly to

5 recombinant viruses presently used to deliver genes to cells for gene therapy and the like, but preferably do not have the ability to express a protein or functional fragment of a protein.

Among useful viruses or viral sequences which may be manipulated to provide the required RNA molecule to the mammalian cell *in vivo* are, without limitation, alphavirus, adenovirus, adeno associated virus, baculoviruses, delta virus, pox viruses, hepatitis viruses, herpes viruses, 10 papova viruses (such as SV40), poliovirus, pseudorabies viruses, retroviruses, lentiviruses, vaccinia viruses, positive and negative stranded RNA viruses, viroids, and virusoids, or portions thereof. These various viral delivery agents may be designed by applying conventional techniques such as described in M. Di Nocla et al., Cancer Gene Ther., 5(6):350-6 (1998), among others, with the teachings of the present invention.

15 The term "*in vivo*" is intended to include any system wherein the cellular DNA or RNA replication machinery is intact, including tissue culture systems, and within single cell or multicellular living organisms.

By "multiple sequitope dsRNA" or "multisequitope dsRNA" is meant an RNA molecule that has segments derived from multiple target nucleic acids or that has non-contiguous segments

20 from the same target nucleic acid. For example, the multiple sequitope dsRNA may have segments derived from (i) sequences representing multiple genes of a single organism; (ii) sequences representing one or more genes from a variety of different organisms; and/or (iii) sequences representing different regions of a particular gene (e.g., one or more sequences from a promoter and one or more sequences from an mRNA). Desirably, each segment has

25 substantial sequence identity to the corresponding region of a target nucleic acid. In various desirable embodiments, a segment with substantial sequence identity to the target nucleic acid is at least 19, 20, 21, 22, 23, 24, 30, 40, 50, 100, 200, 500, 750, or more basepairs in length. In desirable embodiments, the multiple epitope dsRNA inhibits the expression of at least 2, 4, 6, 8, 10, 15, 20, or more target genes by at least 20, 40, 60, 80, 90, 95, or 100%. In some 30 embodiments, the multiple epitope dsRNA has non-contiguous segments from the same target gene that may or may not be in the naturally occurring 5' to 3' order of the segments, and the

dsRNA inhibits the expression of the nucleic acid by at least 50, 100, 200, 500, or 1000% more than a dsRNA with only one of the segments.

By "sequitope" is meant a contiguous sequence of double stranded polyribonucleotides that can associate with and activate RISC (RNA induced silencing complex), usually a contiguous sequence of between 19 and 27 basepairs, inclusive. Sequences comprising at least one sequitope from within one or more of the conserved HBV and/or HCV nucleotide sequences identified here may be utilized for dsRNA mediated gene silencing as taught herein.

Multiple epitope dsRNAs The advantages of a multiple epitope or multisequitope double stranded RNA approach as taught in USSN 60/419,532, filed 18 Oct 2002 and PCT/US2003/033466, filed 20 Oct 30 2003, are applicable to utilization of the conserved HBV and/or HCV sequences of the invention. Because a singular species of dsRNA can simultaneously silence many target genes (e.g., genes from multiple pathogens, multiple genes or sequences from a single pathogen, or genes associated with multiple diseases), a multiple epitope dsRNA can be used for many different indications in the same subject or used for a subset of indications in one subject and another subset of indications in another subject. For such applications, the ability to express long dsRNA molecules (e.g., dsRNA molecules with sequences from multiple genes) without invoking the dsRNA stress response is highly desirable. For example, by using a series of sequences, each, e.g., as short as 19-21 nucleotides, desirably 100 to 600 nucleotides, or easily up to 1, 2, 3, 4, 5, or more kilobases such that the total length of such sequences is within the maximum capacity of the selected plasmid (e.g., 20 kilobases in length), a single such pharmaceutical composition can provide protection against a large number of pathogens and/or toxins at a relatively low cost and low toxicity, e.g., HBV, HCV, HIV, etc.

The use of multiple epitopes derived from one or more genes from multiple strains and/or variants of a highly variable or rapidly mutating pathogen such as HBV and/or HCV can also be very advantageous. For example, a singular dsRNA species that recognizes and targets multiple strains and/or variants of HBV and/or HCV can be used as a universal treatment or vaccine for the various strains/variants of HBV and/or HCV.

The ability to silence multiple genes of a particular pathogen such as HBV and/or HCV prevents the selection of, in this case, HBV and/or HCV "escape mutants." In contrast, typical small molecule treatment or vaccine therapy that only targets one gene or protein results in the selection of pathogens that have sustained mutations in the target gene or protein and the

~~pathogen thus becomes resistant to the therapy. By simultaneously targeting a number of genes or sequences of the pathogen and/or extensive regions of the pathogen using the multiple epitope approach of the present invention, the emergence of such “escape mutants” is effectively precluded.~~

5 ~~For example, it is considered particularly advantageous to include a mixture of sequences from both HCV SEQ ID NO:11 and SEQ ID NO:12, i.e., one or more sequences from HCV SEQ ID NO:11 together with one or more sequences from HCV SEQ ID NO:12, either in a single dsRNA construct, an admixture of constructs, or through concomitant administration of such constructs to a patient, in order to decrease the ability of the virus to generate viable escape~~
 10 ~~mutants. Similarly, it would be advantageous to provide a mixture of the conserved HBV sequences, in some cases in combination with one or more of the conserved HCV sequences of the invention.~~

15 ~~Similarly, it may be desirable to use sequences from two or more of HBV SEQ ID NO:1, SEQ ID NO:2, AND SEQ ID NO:3, either in a single dsRNA construct, an admixture of constructs, or through concomitant administration of such constructs to a patient. SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 map to the HBV surface antigen genes. Due to the overlapping nature of the HBV mRNAs, the following mRNAs would be targeted by one of more of these sequences: Surface Ag (sAg) mRNAs, precore, core and polymerase mRNAs. However, since sAg mRNAs are the most abundant, it is more likely that these mRNAs will be~~
 20 ~~targeted if the gene silencing machinery is saturable. It is possible, however, that all listed mRNAs will be targeted. Reduction of surface Ag is desirable for several reasons: a) surface Ag is needed for assembly of infectious virions; b) overexpression of Surface Ag during infection is thought to contribute to immune anergy that occurs during chronic infection; and c) the expression of sAg in the livers of infected individuals (even in the absence of virus, i.e., from integrated sAg sequences into the host genome) induces hepatitis. Therefore, reduction of sAg is likely to decrease viral titers, overcome immune anergy and decrease/prevent hepatitis.~~
 25 ~~The term “in vivo” is intended to include any system wherein the cellular DNA or RNA replication machinery is intact, preferably within intact living cells, including tissue culture systems, tissue explants, and within single cell or multicellular living organisms.~~

30 By “multiple sequitope dsRNA” or “multisequitope dsRNA” or “multiple epitope dsRNA” is meant an RNA molecule that has segments derived from multiple target nucleic acids

or that has non-contiguous segments from the same target nucleic acid. For example, the multiple sequitope dsRNA may have segments derived from (i) sequences representing multiple genes of a single organism; (ii) sequences representing one or more genes from a variety of different organisms; and/or (iii) sequences representing different regions of a particular gene

5 (e.g., one or more sequences from a promoter and one or more sequences from an mRNA. Desirably, each segment has substantial sequence identity to the corresponding region of a target nucleic acid. In various desirable embodiments, a segment with substantial sequence identity to the target nucleic acid is at least 19, 20, 21, 22, 23, 24, 25, 26, 27, 30, 40, 50, 100, 200, 500, 750, or more basepairs in length. In desirable embodiments, the multiple epitope dsRNA inhibits the expression of at least 2, 4, 6, 8, 10, 15, 20, or more target genes by at least 20, 40, 60, 80, 90, 95, or 100%. In some embodiments, the multiple epitope dsRNA has non-contiguous segments from the same target gene or from the same target polynucleotide that may or may not be in the naturally occurring 5' to 3' order of the segments, and the dsRNA inhibits the expression of the target nucleic acid by at least 50, 100, 200, 500, or 1000% more than a dsRNA with only one of the segments.

10 By "sequitope" is meant a contiguous sequence of double-stranded polyribonucleotides that can associate with and activate RISC (RNA-induced silencing complex), usually a contiguous sequence of between 19 and 27 basepairs, inclusive. Sequences comprising at least one sequitope from within one or more of the conserved HBV and/or HCV nucleotide sequences identified here may be utilized for dsRNA mediated gene silencing as taught herein.

15 Multiple-epitope/ multiple-sequitope dsRNAs the advantages of a multiple-epitope or multisequitope double-stranded RNA approach as taught in USSN 60/419,532, filed 18-Oct-2002 and PCT/US2003/033466, filed 20-Oct-2003, are applicable to utilization of the conserved HBV and/or HCV sequences of the invention. Because a singular species of dsRNA can simultaneously silence many target genes (e.g., genes from multiple pathogens, multiple genes or sequences from a single pathogen, or genes associated with multiple diseases), a multiple epitope dsRNA can be used for many different indications in the same subject or used for a subset of indications in one subject and another subset of indications in another subject. For such applications, the ability to express long dsRNA molecules (e.g., dsRNA molecules with sequences from multiple genes) without invoking the dsRNA stress response is highly desirable. For example, by using a series of sequences, each, e.g., as short as 19-21 nucleotides, desirably

100 to 600 nucleotides, or easily up to 1, 2, 3, 4, 5, or more kilobases such that the total length of such sequences is within the maximum capacity of the selected plasmid (e.g., 20 kilobases in length), a single such pharmaceutical composition can provide protection against a large number of pathogens and/or toxins at a relatively low cost and low toxicity, e.g., HBV, HCV, HIV, etc.

5 The use of multiple epitopes or sequitopes derived from one or more genes and/or different overlapping and/or non-contiguous sequences of the same polynucleotide or gene from multiple strains and/or variants of a highly variable or rapidly mutating pathogen such as HBV and/or HCV can also be very advantageous. For example, a singular dsRNA species that recognizes and targets multiple strains and/or variants of HBV and/or HCV can be used as a

10 universal treatment or vaccine for the various strains/variants of HBV and/or HCV.

15 The ability to silence multiple genes of a particular pathogen such as HBV and/or HCV prevents the selection of, in this case, HBV and/or HCV “escape mutants.” In contrast, typical small molecule treatment or vaccine therapy that only targets one gene or protein results in the selection of pathogens that have sustained mutations in the target gene or protein and the pathogen thus becomes resistant to the therapy. By simultaneously targeting a number of genes or sequences of the pathogen and/or extensive regions of the pathogen using the multiple epitope approach of the present invention, the emergence of such “escape mutants” is effectively precluded.

20 For example, it is considered particularly advantageous to include a mixture of sequences from both HCV SEQ ID NO:11 and SEQ ID NO:12, and SEQ ID NO: 27, i.e., one or more sequences (e.g, each at least 19, 20, 21, 22, 23, 24, 25, 26, 27 to 29 contiguous nucleotides) from HCV SEQ ID NO:11 together with one or more sequences (e.g, each at least 19, 20, 21, 22, 23, 24, 25, 26, 27 to 29 contiguous nucleotides) from HCV SEQ ID NO:12 and from SEQ ID NO: 27 , either in a single dsRNA molecule, an admixture of dsRNA molecules, or through

25 concomitant administration of such molecules to a patient (or by administering one or more dsRNA expression constructs which produce such dsRNA molecules intracellularly), in order to decrease the ability of the virus to generate viable escape mutants. Similarly, it would be advantageous to provide a mixture of dsRNA molecules comprising a number of the conserved HBV sequences, in some cases in combination with one or more of the conserved HCV sequences of the invention.

Similarly, it may be desirable to use sequences from two or more of HBV SEQ ID NO:1, SEQ ID NO:2, AND SEQ ID NO:3, either in a single dsRNA construct, an admixture of constructs, or through concomitant administration of such constructs (or dsRNA expression constructs which produce such dsRNA molecules) to a patient. SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 map to the HBV surface antigen genes. Due to the overlapping nature of the HBV mRNAs, the following mRNAs would be targeted by one of more of these sequences: Surface Ag (sAg) mRNAs, precore, core and polymerase mRNAs. However, since sAg mRNAs are the most abundant, it is more likely that these mRNAs will be targeted if the gene-silencing machinery is saturable. It is possible, however, that all listed mRNAs will be targeted.

10 Reduction of surface Ag is desirable for several reasons: a) surface Ag is needed for assembly of infectious virions; b) overexpression of Surface Ag during infection is thought to contribute to immune anergy that occurs during chronic HBV infection; and c) the expression of HBVsAg in the livers of infected individuals (even in the absence of virus, i.e., from integrated sAg sequences into the host genome) induces hepatitis. Therefore, reduction of sAg is likely to

15 decrease viral titers, overcome immune anergy and decrease/prevent hepatitis.

HBV SEQ ID NO:4 maps to the unique region of precore and core and will target these mRNAs specifically. Core protein is needed to make functional virions and so down regulation of this mRNA is predicted to decrease viral titers. There should be no competition of these effector RNAs for surface, polymerase or X mRNAs.

20 HBV SEQ ID NO:5 through SEQ ID NO:8 map to the polymerase gene. Effector RNAs are predicted to target only precore/core and polymerase transcripts. There should be no competition with sAg or X mRNAs. Polymerase is needed for the synthesis of viral genomes and therefore viral particle titer is expected to decrease if polymerase is decreased.

25 HBV SEQ ID NO:9 maps to the X gene. Due to the terminal redundancy of all mRNAs, these effector RNAs have the potential to target all viral mRNAs. X protein has many ascribed (non proven) functions. However, the X gene is often found in integrated HBV sequences in individuals with active hepatitis and down regulation of X gene expression is predicted to ameliorate disease.

30 In general, the more sequences from the different identified sequences that are used (e.g., from SEQ ID NO:1, SEQ ID NO:2, and/or SEQ ID NO:3, plus sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID

NO:10), the less likely a virus will be able to generate viable escape mutants. Also, the more different mRNAs that can be targeted, the more significant will be the drops in viral titer and disease amelioration.

Desirable combinations for multiepitope constructs, an admixture of constructs, or the concomitant administration of different dsRNA constructs include the following: Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:5; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9 and SEQ ID NO:10.

NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9 and SEQ ID NO:10; Sequences from SEQ ID NO:4 and SEQ ID NO:5; Sequences from SEQ ID NO:4 and SEQ ID NO:6; Sequences from SEQ ID NO:4 and SEQ ID NO:7; Sequences from SEQ ID NO:4 and SEQ ID NO:8; Sequences from SEQ ID NO:4 and SEQ ID NO:9; Sequences from SEQ ID NO:4 and SEQ ID NO:10; Sequences from SEQ ID NO:5 and SEQ ID NO:6; Sequences from SEQ ID NO:5 and SEQ ID NO:7; Sequences from SEQ ID NO:5 and SEQ ID NO:8; Sequences from SEQ ID NO:5 and SEQ ID NO:9; Sequences from SEQ ID NO:5 and SEQ ID NO:10; Sequences from SEQ ID NO:6 and SEQ ID NO:7; Sequences from SEQ ID NO:6 and SEQ ID NO:8; Sequences from SEQ ID NO:6 and SEQ ID NO:9; Sequences from SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:7 and SEQ ID NO:9; Sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences from SEQ ID NO:9 and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:6; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6; and

~~SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; and Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10;~~

5 ~~SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10.~~

~~In an another embodiment, combinations of sequitopes and longer sequences from within any of the aforementioned sequences (e.g., SEQ ID NO:1 through SEQ ID NO:12) may be utilized either in a single dsRNA construct, an admixture of constructs, or through concomitant administration of such constructs to a patient.~~

10 As discussed elsewhere herein, a particularly preferred embodiment of the invention utilizes dsRNA expression constructs or vectors to achieve endogenous delivery of the dsRNAs of the invention, especially the multiple different sequences described above. These dsRNAs may be provided e.g., on the same cistron of an expression construct such as a plasmid, on 15 different cistrons of an expression construct, or on different expression constructs or plasmids, e.g., one or more plasmids and/or one or more vectors, including viral vectors. Such multiple different sequences may also be provided exogenously, in any different mixture of one or more dsRNA structures, duplexes and/or harpins, and/or in combination with one or more endogenously expressed dsRNA structures.

20 Desirable methods of administration of nucleic acids The DNA and/or RNA constructs of the invention may be administered to the host cell/tissue/organism as "naked" DNA, RNA, or DNA/RNA, formulated in a pharmaceutical vehicle without any transfection promoting agent. More efficient delivery may be achieved as known to those of skill in the art of DNA and RNA delivery, using e.g., such polynucleotide transfection facilitating agents known to those of skill in 25 the art of RNA and/or DNA delivery. The following are exemplary agents: cationic amphiphiles including local anesthetics such as bupivacaine, cationic lipids, liposomes or lipidic particles, polycations such as polylysine, branched, three dimensional polycations such as dendrimers, carbohydrates, detergents, or surfactants, including benzylammonium surfactants such as benzalkonium chloride. Non exclusive examples of such facilitating agents or co-agents useful 30 in this invention are described in U.S. Patent numbers 5,593,972; 5,703,055; 5,739,118; 5,837,533; 5,962,482; 6,127,170; 6,379,965; and 6,482,804; and International Patent Application

~~No. PCT/US98/22841; the teaching of which is hereby incorporated by reference. U.S. Patents numbers 5,824,538; 5,643,771; and 5,877,159 (incorporated herein by reference) teach delivery of a composition other than a polynucleotide composition, e.g., a transfected donor cell or a bacterium containing the dsRNA encoding compositions of the invention.~~

HBV SEQ ID NO:5 through SEQ ID NO:8 map to the polymerase gene. Effector RNAs are predicted to target only precore/core and polymerase transcripts. There should be no competition with sAg or X mRNAs. Polymerase is needed for the synthesis of viral genomes and therefore viral particle titer is expected to decrease as polymerase is decreased.

HBV SEQ ID NO:9 maps to the X gene. Due to the terminal redundancy of all the HBV mRNAs, these effector RNAs have the potential to target all of the HBV viral mRNAs. X protein has many ascribed (non proven) functions. Evidence is emerging, however, that X-gene expression is associated with hepatocellular carcinogenesis, in part related to promotion of detachment and migration of cells out of the primary tumor site. Since the X gene is often found in integrated HBV sequences in individuals with and without active hepatitis, down-regulation of X gene expression is predicted to ameliorate disease, including the incidence of hepatocellular carcinoma.

In general, the more sequences or sequitopes from the different identified sequences that are used (e.g., from SEQ ID NO:1, SEQ ID NO:2, and/or SEQ ID NO:3, plus sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10), the less likely a virus will be able to generate viable escape mutants. Also, the more different mRNAs that can be targeted, the more significant will be the drops in viral titer and disease amelioration.

Desirable combinations for multiepitope or multisequitope dsRNA expression constructs
or dsRNA effector molecules, an admixture of dsRNA expression constructs or dsRNA effector
25 molecules , or the concomitant administration of different dsRNA expression constructs or
dsRNA effector molecules include the following: Sequences from SEQ ID NO:1, SEQ ID NO:2,
or SEQ ID NO:3 plus sequences from SEQ ID NO:4; Sequences from SEQ ID NO:1, SEQ ID
NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5; Sequences from SEQ ID NO:1,
SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6; Sequences from SEQ ID
30 NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7; Sequences from
SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8; Sequences

from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9;
Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID
NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from
SEQ ID NO:4 and SEQ ID NO:5; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID

and SEQ ID NO:8; Sequences from SEQ ID NO:4 and SEQ ID NO:9; Sequences from SEQ ID NO:4 and SEQ ID NO:10; Sequences from SEQ ID NO:5 and SEQ ID NO:6; Sequences from SEQ ID NO:5 and SEQ ID NO:7; Sequences from SEQ ID NO:5 and SEQ ID NO:8; Sequences from SEQ ID NO:5 and SEQ ID NO:9; Sequences from SEQ ID NO:5 and SEQ ID NO:10;

5 Sequences from SEQ ID NO:6 and SEQ ID NO:7; Sequences from SEQ ID NO:6 and SEQ ID NO:8; Sequences from SEQ ID NO:6 and SEQ ID NO:9; Sequences from SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:7 and SEQ ID NO:9; Sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences

10 from SEQ ID NO:9 and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:6; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:7; Sequences from SEQ ID

15 NO:4, SEQ ID NO:6; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID

20 NO:4, SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:7; Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:8; Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID

25 NO:5, SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:9; Sequences from SEQ ID NO:6, SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:6, SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID

30 NO:6, SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:6, SEQ ID NO:8; and SEQ ID NO:10; Sequences

Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:9, and

5 SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8,
and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8,
and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID
NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ
ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ
10 ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7,
SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID
NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID
NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ
ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6,
15 SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID
NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ
ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4,
SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from
SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10;
20 Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and
SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8,
SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID
NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ
25 ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from
SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10;
and Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8,
SEQ ID NO:9, and SEQ ID NO:10. Preferred in some aspects are sequences from SEQ ID
NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8, including combinations of sequitopes
from SEQ ID NO:5, plus SEQ ID NO:6, plus SEQ ID NO:7, plus SEQ ID NO:8

30 In another embodiment, combinations of sequitopes at least 19 contiguous base pairs in length and longer sequences from within any of the aforementioned sequences (e.g., SEQ ID

NO:1 through SEQ ID NO:12) may be utilized either in a single dsRNA expression construct or dsRNA effector molecule, an admixture of dsRNA expression constructs or dsRNA effector molecules or through concomitant administration of such dsRNA expression constructs or dsRNA effector molecules to a patient. By a sequence of “at least 19 contiguous base pairs in length” is meant that a sequence or sequitope of at 19 contiguous bases in length is present in double-stranded conformation, or within a double-stranded RNA effector molecule.

As discussed elsewhere herein, a particularly preferred embodiment of the invention utilizes dsRNA expression constructs or vectors to achieve endogenous delivery of the dsRNAs of the invention, especially the multiple different sequences described above. These dsRNAs may be provided e.g., on the same cistron of an expression construct such as a plasmid, on different cistrons of an expression construct, or on different expression constructs or plasmids, e.g., one or more plasmids and/or one or more vectors, including viral vectors. The combination of different dsRNA effector molecules such as shRNA effector molecules may be provided to a mammalian cell by in vivo expression from a single expression construct such as a plasmid, with each dsRNA effector molecule transcribed from a different expression cassette driven by a different promoter, e.g., an RNA polymerase I promoter and/or an RNA polymerase III promoter, e.g., a type 3 RNA polymerase III promoter such as U6, H1, 7SK, or MRP. In some embodiments, each such different expression cassette may contain a different RNA polymerase III promoter, which may be the same or different, and an RNA polymerase III termination sequence. In another embodiment, a combination of different dsRNA effector molecules such as shRNA effector molecules may be provided to a mammalian cell by in vivo expression from a single expression construct such as a plasmid or a viral vector which comprise an expression cassette comprising multiple different promoters, e.g., an RNA polymerase I promoter and/or an RNA polymerase III promoter, e.g., a type 3 RNA polymerase III promoter such as U6, H1, 7SK, or MRP, and wherein each of such promoters transcribes a different dsRNA effector molecule. Such multiple different dsRNA effector sequences may also be provided to an in vivo mammalian cell exogenously, in any different mixture of one or more dsRNA structures, duplexes and/or harpins, and/or in combination with one or more endogenously expressed dsRNA structures.

Desirable methods of administration of nucleic acids The DNA and/or RNA constructs, e.g., dsRNA effector molecules, of the invention may be administered to the host

cell/tissue/organism as “naked” DNA, RNA, or DNA/RNA, formulated in a pharmaceutical vehicle without any transfection promoting agent. More efficient delivery may be achieved as known to those of skill in the art of DNA and RNA delivery, using e.g., such polynucleotide transfection facilitating agents known to those of skill in the art of RNA and/or DNA delivery.

- 5 The following are exemplary agents: cationic amphiphiles including local anesthetics such as bupivacaine, cationic lipids, liposomes or lipidic particles, polycations such as polylysine, branched, three-dimensional polycations such as dendrimers, carbohydrates, detergents, or surfactants, including benzylammonium surfactants such as benzalkonium chloride. Non-exclusive examples of such facilitating agents or co-agents useful in this invention are described
- 10 in U.S. Patent numbers 5,593,972; 5,703,055; 5,739,118; 5,837,533; 5,962,482; 6,127,170; 6,379,965; and 6,482,804; and International Patent Application No. PCT/US98/22841; the teaching of which is hereby incorporated by reference. U.S. Patents numbers 5,824,538; 5,643,771; and 5,877,159 (incorporated herein by reference) teach delivery of a composition other than a polynucleotide composition, e.g., a transfected donor cell or a bacterium containing
- 15 the dsRNA-encoding compositions of the invention.

In some embodiments, the dsRNA or dsRNA expression vector is complexed with one or more cationic lipids or cationic amphiphiles, such as the compositions disclosed in US 4,897,355 (Eppstein *et al.*, filed October 29, 1987), US 5,264,618 (Feigner *et al.*, filed April 16, 1991) or US 5,459,127 (Feigner *et al.*, filed September 16, 1993). In other embodiments, the dsRNA or dsRNA expression vector is complexed with a liposome/liposomal composition that includes a cationic lipid and optionally includes another component such as a neutral lipid (see, for example, US 5,279,833 (Rose), US 5,283,185 (Epand), and US 5,932,241).

Particularly desirable methods and compositions for delivery of the oligonucleotide compositions of the invention for pharmaceutical applications, including for targeted delivery to hepatocytes, are described in PCT/US03/14288, filed May 6, 2003, the teaching of which is incorporated herein by reference.

Transformation/transfection of the cell for research and other non-therapeutic purposes may occur through a variety of means including, but not limited to, lipofection, DEAE-dextran-mediated transfection, microinjection, calcium phosphate precipitation, viral or retroviral delivery, electroporation, or biolistic transformation. The RNA or RNA expression vector (DNA) may be naked RNA or DNA or local anesthetic complexed RNA or DNA (See U.S.

Patent Nos. 6,217,900 and 6,383,512, "Vesicular Complexes and Methods of Making and Using the Same, Pachuk *et al.*, *supra*).

Another desirable delivery technology for the dsRNAs or dsRNA expression constructs of the invention for pharmaceutical applications is the self-assembling Cyclosert™ two-component nucleic acid delivery system, based on cyclodextrin-containing polycations, which are available from Insert Therapeutics, Pasadena, CA. (See Bioconjug Chem 2003 May-Jun; 14 (3): 672-8; Popielarski et al.; "Structural effects of carbohydrate-containing polycations on gene delivery. 3. Cyclodextrin type and functionalization"; as well as Bioconjug Chem 2003 Jan-Feb; 14 (1):247-54 and 255-61.) The first component is a linear, cyclodextrin-containing polycationic polymer, that when mixed with DNA, binds to the phosphate "backbone" of the nucleic acid, condensing the DNA and self assembling into uniform, colloidal nanoparticles that protect the DNA from nuclease degradation in serum. A second component is a surface modifying agent with a terminal adamantine-PEG molecule, that when combined with the cyclodextrin polymer forms an inclusion complex with surface cyclodextrins and prevents aggregation, enhances stability and enables systemic administration. In addition, targeting ligands to cell surface receptors may be attached to the modifier providing for targeted delivery of DNA directly to target cells of interest. Since hepatocytes are susceptible to HBV and HCV infection, utilizing this method to target delivery of the dsRNA expression constructs of the invention to liver cells is considered especially advantageous. E.g., the asialoglycoprotein receptor (ASGP-R) on mammalian hepatocytes may be targeted by use of synthetic ligands with galactosylated or lactosylated residues, such as galactosylated polymers.

~~In general, targeting for selective delivery of the dsRNA constructs of the invention to hepatocytes is preferred. Targeting to hepatocytes may be achieved by coupling to ligands for hepatocyte specific receptors. For example, asialo-orosomucoid, (poly)L lysine asialo-orosomucoid, or any other ligands of the hepatic asialoglycoprotein receptor (Spiess, Biochemistry 29(43):10009-10018, 1990; Wu et al., J. Biol. Chem. 267(18):12436-12439, 1992; Wu et al., Biotherapy 3:87-95, 1991). Similarly, the oligonucleotides may be targeted to hepatocytes by being conjugated to monoclonal antibodies that specifically bind to hepatocyte specific receptors. Oligonucleotides may also be targeted to hepatocytes using specific vectors, as described below.~~

In general, targeting for selective delivery of the dsRNA constructs of the invention to hepatocytes is preferred. Targeting to hepatocytes may be achieved by coupling to ligands for hepatocyte-specific receptors. For example, asialo-orosomucoid, (poly)L-lysine-asialo-orosomucoid, or any other ligands of the hepatic asialoglycoprotein receptor (Spiess,

- 5 Biochemistry 29(43):10009-10018, 1990; Wu et al., J. Biol. Chem. 267(18):12436-12439, 1992;
Wu et al., Biotherapy 3:87-95, 1991). Similarly, the oligonucleotides may be targeted to hepatocytes by being conjugated to monoclonal antibodies that specifically bind to hepatocyte-specific receptors. Oligonucleotides may also be targeted to hepatocytes using specific vectors, as described below.

- 10 Particularly preferred compositions for delivery of dsRNAs or dsRNA expression constructs of the invention are the multifunctional compositions as described in PCT/US03/14288, filed May 6, 2003, which may include trilactosyl spermine as a ligand for targeting to the ASG Receptor of hepatocytes. Trilactosyl cholesteryl spermine co-complexes with the oligonucleotides of the invention may be prepared and used as described to transfect 15 hepatocytes *in vivo*.

The dsRNA oligonucleotides of the invention may be provided exogenously to a target hepatocyte. Alternatively, a dsRNA may be produced within the target cell by transcription of a nucleic acid molecule comprising a promoter sequence operably linked to a sequence encoding the dsRNA. In this method, the nucleic acid molecule is contained within a non replicating 20 linear or circular DNA or RNA molecule, or is contained within an autonomously replicating plasmid or viral vector, or is integrated into the host genome. Any vector that can transfect a hepatocyte may be used in the methods of the invention. Preferred vectors are viral vectors, including those derived from replication defective hepatitis viruses (e.g., HBV and HCV), retroviruses (see, e.g., WO89/07136; Rosenberg et al., N. Eng. J. Med. 323(9):570-578, 1990), 25 adenovirus (see, e.g., Morsey et al., J. Cell. Biochem., Supp. 17E, 1993; Graham et al., in Murray, ed., Methods in Molecular Biology: Gene Transfer and Expression Protocols. Vol. 7, Clifton, N.J.: the Human Press 1991: 109-128), adeno associated virus (Kotin et al., Proc. Natl. Acad. Sci. USA 87:2211-2215, 1990), replication defective herpes simplex viruses (HSV; Lu et al., Abstract, page 66, Abstracts of the Meeting on Gene Therapy, Sep. 22-26, 1992, Cold Spring 30 Harbor Laboratory, Cold Spring Harbor, N.Y.), and any modified versions of these vectors. Methods for constructing expression vectors are well known in the art (see, e.g., Molecular

Cloning: A Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, N.Y., 1989).

The dsRNA oligonucleotides of the invention may be provided exogenously to a target hepatocyte, e.g., prepared outside the cell and delivered into a mammalian hepatocyte.

- 5 Alternatively, a dsRNA may be produced within the target cell by transcription of a nucleic acid molecule comprising a promoter sequence operably linked to a sequence encoding the dsRNA. In this method, the nucleic acid molecule is contained within a non-replicating linear or circular DNA or RNA molecule, or is contained within an autonomously replicating plasmid or viral vector, or is integrated into the host genome. Any vector that can transfect a hepatocyte may be
- 10 used in the methods of the invention. Preferred vectors are viral vectors, including those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses (see, e.g., W089/07136; Rosenberg et al., N. Eng. J. Med. 323(9):570-578, 1990), adenovirus (see, e.g., Morsey et al., J. Cell. Biochem., Supp. 17E, 1993; Graham et al., in Murray, ed., Methods in Molecular Biology: Gene Transfer and Expression Protocols. Vol. 7, Clifton, N.J.: the Human Press 1991: 109-128), adeno-associated virus (Kotin et al., Proc. Natl. Acad. Sci. USA 87:2211-
- 15 2215, 1990), replication defective herpes simplex viruses (HSV; Lu et al., Abstract, page 66, Abstracts of the Meeting on Gene Therapy, Sep. 22-26, 1992, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), and any modified versions of these vectors. Methods for constructing expression vectors are well known in the art (see, e.g., Molecular Cloning: A
- 20 Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, N.Y., 1989).

Appropriate regulatory sequences can be inserted into the vectors of the invention using methods known to those skilled in the art, for example, by homologous recombination (Graham et al., J. Gen. Virol. 36:59-72, 1977), or other appropriate methods (Molecular Cloning: A Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, N.Y., 1989).

- Upon assembly of a recombinant DNA plasmid dsRNA expression vector on the invention, bacteria are used as "factories" to produce large quantities of the final vector. The E.coli bacterium is frequently used for plasmid fermentation, and it may be advantageous to employ for this purpose E. coli strains having a reduced genome as described in, e.g., Blattner et al., Published U.S. Patent Application No. 2005/0032225, the teaching of which is incorporated

herein by reference. The vector manufactured in this manner, isolated and purified according to methods known in the art, can be introduced into living cells with a variety of methods, collectively known as “transfection”, including the methods and compositions described above. Once inside the cell, the promoter elements are recognized by the cellular machinery available

5 for gene transcription and the RNA effector molecules, e.g., shRNAs, will be produced.

Other bacterial strains that may be advantageous for propagating a plasmid expression vector of the invention include the E. coli GT116 Competent Cells available commercially from InvivoGen, San Diego, CA. GT116 is a sbcCD deletion strain specifically engineered to support the growth of plasmid DNAs carrying hairpin structures, such as the plasmids of the invention

10 engineered to express one or more dsRNA effector molecules which are hairpin RNAs. Hairpin structures are known to be unstable in E. coli due to their elimination by a protein complex called SbcCD that recognizes and cleaves hairpins (Connelly et al., Proc. Natl. Acad. Sci. USA 95:7969-74 (1998)). The sbcCD and sbcD genes are deleted in E. coli GT116, which improves its utility for cloning plasmids with hairpin or other palindrome-containing structures.

15 Promoters Promoters are inserted into the vectors so that they are operably linked, typically but not invariably, 5' to the nucleic acid sequence encoding the dsRNA oligonucleotide. Any promoter that is capable of directing initiation of transcription in a eukaryotic cell may be used in the invention. For example, non-tissue-specific promoters, such 20 as the cytomegalovirus (DeBernardi et al., Proc. Natl. Acad. Sci. USA 88:9257-9261, 1991, and references therein), mouse metallothioneine I gene (Hammer, et al., J. Mol. Appl. Gen. 1:273-288, 1982), HSV thymidine kinase (McKnight, Cell 31:355-365 1982), and SV40 early (Benoist et al., Nature 290:304-310, 1981) promoters may be used. Non-tissue-specific promoters may be used in the invention, as expression of HBV and/or HCV dsRNA in non-liver cells directed by 25 the non-tissue-specific promoters should be harmless to the non-liver cells, because of the specificity of the HBV and HCV dsRNAs of the invention for viral sequences. However, preferred promoters for use in the invention are hepatocyte-specific promoters, the use of which ensures that the RNAs are expressed primarily in hepatocytes. Preferred hepatocyte-specific promoters include, but are not limited to the albumin, alpha-fetoprotein, alpha-1-antitrypsin, 30 retinol-binding protein, and asialoglycoprotein receptor promoters. Viral promoters and enhancers, such as those from cytomegalovirus, herpes simplex viruses (types I and II), hepatitis

viruses (A, B, and C), and Rous sarcoma virus (RSV; Fang et al., Hepatology 10:781-787, 1989), may also be used in the invention.

dsRNA expression vectors may include promoters for RNA polymerase I, RNA polymerase II including but not limited to HCMV, SCMV, MCMV, RSV, EF2a, TK and other 5 HSV promoters such as ICP6, ICP4 and ICP0 promoters, HBV pregenomic promoter, RNA pol III promoter including but not limited to U6 and tRNA promoters, mitochondrial light and heavy strand promoters. Desirably, the dsRNA expression vector comprises at least one RNA polymerase II promoter, for example, a human CMV immediate early promoter (HCMV IE) or a simian CMV (SCMV) promoter, at least one RNA polymerase I promoter, or at least one RNA 10 polymerase III promoter. The promoter may also be a T7 promoter, in which case, the cell further comprises T7 RNA polymerase. Alternatively, the promoter may be an SP6 promoter, in which case, the cell further comprises SP6 RNA polymerase. The promoter may also be one convergent T7 promoter and one convergent SP6 RNA promoter. A cell may be made to contain T7 or SP6 polymerase by transforming the cell with a T7 polymerase or an SP6 polymerase 15 expression plasmid, respectively. In some embodiments, a T7 promoter or a RNA polymerase III promoter is operably linked to a nucleic acid that encodes a short dsRNA (e.g., a dsRNA that is less than 200, 150, 100, 75, 50, or 25 basepairs in length). In other embodiments, the promoter is a mitochondrial promoter that allows cytoplasmic transcription of the nucleic acid in the vector (see, for example, the mitochondrial promoters described in WO 00/63364, filed April 19, 2000, 20 and in WO/US2002/00543, filed 9 Jan 2001). Alternatively, the promoter is an inducible promoter, such as a *lac* (Cronin et al. Genes & Development 15: 1506-1517, 2001), *ara* (Khlebnikov et al., J Bacteriol. 2000 Dec;182(24):7029-34), ecdysone (Rheogene website), RU48 (mifepristone) (corticosteroid antagonist) (Wang XJ, Liefer KM, Tsai S, O'Malley BW, Reop DR, Proc Natl Acad Sci U S A. 1999 Jul 20;96(15):8483-8), or *tet* promoter (Rendal et al., 25 Hum Gene Ther. 2002;13(2):335-42 and Larnartina et al., Hum Gene Ther. 2002;13(2):199-210) or a promoter disclosed in WO 00/63364, filed April 19, 2000. Also useful in the methods and compositions of the invention are the structural and chimeric promoters taught in USSN 60/464,434, filed 22 Apr 2003. See also the promoter systems taught in Pachuk, C., and Satishchandran, C., "Multiple Compartment Eukaryotic Expression Systems," U.S. Provisional 30 Application No. 60/497,304, filed 22 Aug 2003, which are considered particularly desirable in the methods and compositions of the invention.

dsRNA expression vectors may include promoters for RNA polymerase I, RNA polymerase II including but not limited to HCMV, SCMV, MCMV, RSV, EF2a, TK and other HSV promoters such as ICP6, ICP4 and ICP0 promoters, HBV pregenomic promoter, RNA pol III promoter, especially type 3 RNA polymerase III promoters, including but not limited to 7SK,

5 U6, and H1, and tRNA promoters, as well as mitochondrial light and heavy strand promoters.

Desirably, the dsRNA expression vector comprises at least one RNA polymerase II promoter, for example, a human CMV-immediate early promoter (HCMV-IE) or a simian CMV (SCMV) promoter, at least one RNA polymerase I promoter, or at least one RNA polymerase III promoter. The promoter may also be a T7 promoter, in which case, the cell further comprises T7

10 RNA polymerase. Alternatively, the promoter may be an SP6 promoter, in which case, the cell further comprises SP6 RNA polymerase. The promoter may also be one convergent T7 promoter and one convergent SP6 RNA promoter. A cell may be made to contain T7 or SP6 polymerase by transforming the cell with a T7 polymerase or an SP6 polymerase expression plasmid, respectively. In some embodiments, a T7 promoter or a RNA polymerase III promoter

15 is operably linked to a nucleic acid that encodes a short dsRNA (e.g., a dsRNA that is less than 200, 150, 100, 75, 50, or 25 basepairs in length). In other embodiments, the promoter is a mitochondrial promoter that allows cytoplasmic transcription of the nucleic acid in the vector (see, for example, the mitochondria) promoters described in WO 00/63364, filed April 19, 2000, and in WO/US2002/00543, filed 9-Jan-2001). Alternatively, the promoter is an inducible

20 promoter, such as a lac (Cronin et al. Genes & Development 15: 1506-1517, 2001), ara (Khlebnikov et al., J Bacteriol. 2000 Dec;182(24):7029-34), ecdysone (Rheogene website), RU48 (mefepristone) (corticosteroid antagonist) (Wang XJ, Liefer KM, Tsai S, O'Malley BW, Roop DR, Proc Natl Acad Sci U S A. 1999 Jul 20;96(15):8483-8), or tet promoter (Rendal et al., Hum Gene Ther. 2002;13(2):335-42 and Larnartina et al., Hum Gene Ther. 2002;13(2):199-210)

25 or a promoter disclosed in WO 00/63364, filed April 19, 2000. Also useful in the methods and compositions of the invention are the structural and chimeric promoters taught in USSN 60/464,434, filed 22-Apr-2003. See also the promoter systems taught in Pachuk, C., and Satishchandran, C., "Multiple-Compartment Eukaryotic Expression Systems," U.S. Provisional Application No. 60/497,304, filed 22-Aug-2003, which are considered particularly desirable in

30 the methods and compositions of the invention.

Liver specific promoters useful in dsRNA expression constructs of the invention include the albumin promoter, the alpha-fetoprotein promoter (especially in liver cancer cells), the alpha-1-antitrypsin promoter, hepatitis B promoters, e.g., hepatitis B promoters including promoters for the antigen genes, including core, e antigen, polymerase and X protein.

- 5 T7 Promoter/T7 Polymerase Expression Systems A desirable method of the invention utilizes a T7 dsRNA expression system to achieve cytoplasmic expression of dsRNA, (e.g., long or short dsRNA molecules) in vertebrate cells (e.g., mammalian cells). The T7 expression system utilizes the T7 promoter to express the desired dsRNA. Transcription is driven by the T7 RNA polymerase, which can be provided on a second plasmid or on the same plasmid.
- 10 Bacteriophage T7 RNA polymerase (T7 Pol) is the product of T7 gene 1, which can recognize its responsive promoter sequence specifically and exhibit a high transcriptase activity. The complete sequence of the T7 genome, with detailed information about the different regions of the bacteriophage, including promoter sequences, is disclosed in Dunn & Studier, 1983, J. Mol. Biol. 166(4), 477-535 (see also NCBI ‘Genome’ database, Accession No. NC 001604). The T7
- 15 promoter cannot be utilised by any other RNA polymerase than the polymerase of bacteriophage T7, which shows a stringent specificity for the promoter (Chamberlin et al., 1970, Nature 228:227-231). When utilizing the T7 expression system for expressing dsRNAs, for example, a first plasmid construct that expresses both a sense and antisense strand under the control of converging T7 promoters and a second plasmid construct that expresses the T7 RNA polymerase
- 20 under the control of an RSV promoter can be used. Both the dsRNA and the T7 RNA polymerase could advantageously be expressed from a single bicistronic plasmid construct, particularly when the dsRNA is formed from a single RNA strand with inverted repeats or regions of self-complementarity that enable the strand to assume a stem-loop or hairpin structure with an at least partially double stranded region. Individual sense and antisense strands which
- 25 self assemble to form a dsRNA can be synthesized by a single plasmid construct using, e.g., converging promoters such as bacteriophage T7 promoters placed respectively at the 5' and 3' ends of the complementary strands of a selected sequence to be transcribed. See also, e.g., the teaching of WO 0063364, with respect to T7 dsRNA expression systems, as well as USSN 60/399,998P, filed 31-Jul-2002 and USSN 60/419,532, filed 18-Oct-2002.
- 30 Therapeutic Compositions of the Invention The dsRNAs of the invention, and the recombinant vectors containing nucleic acid sequences encoding them, may be used in

therapeutic compositions for preventing or treating HCV and/or HBV infection. The therapeutic compositions of the invention may be used alone or in admixture, or in chemical combination, with one or more materials, including other antiviral agents. Currently, lamivudine, adefovir dipivoxil, and interferon alpha have been approved for treatment of HBV, and it is anticipated
5 that the compositions of the invention may be used in combination with these and other drugs that are active against HBV, including emtricitabine (FTC) and entecavir. Since dsRNAs against HBV and/or HCV act through a novel mechanism (dsRNA-mediated gene silencing/RNAi), combination therapy of the agents of the invention and other antivirals is expected to significantly increase the efficacy of therapy while substantially reducing the development of
10 drug resistance, e.g., the development of lamivudine resistance, a problem of major concern with long term lamivudine therapy. Currently, interferon and ribavirin are licensed for treatment of HCV, and as for HBV, it is anticipated that the compositions of the invention may be used in combination with these and other drugs that are active against HCV. Specific dosage regimens involving therapy with such multiple agents can be determined through routine experimentation
15 by those of ordinary skill in the art of clinical medicine.

Formulations will desirably include materials that increase the biological stability of the oligonucleotides or the recombinant vectors, or materials that increase the ability of the therapeutic compositions to penetrate hepatocytes selectively. The therapeutic compositions of the invention may be administered in pharmaceutically acceptable carriers (e.g., physiological saline), which are selected on the basis of the mode and route of administration, and standard pharmaceutical practice. One having ordinary skill in the art can readily formulate a pharmaceutical composition that comprises an oligonucleotide or a gene construct. In some cases, an isotonic formulation is used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vasoconstriction agent is added to the formulation. The pharmaceutical preparations according to the present invention are provided sterile and pyrogen free. Suitable pharmaceutical carriers, as well as pharmaceutical necessities for use in pharmaceutical formulations, are described in Remington: The Science and Practice of Pharmacy (formerly
20 Remington's Pharmaceutical Sciences), Mack Publishing Co., a standard reference text in this field, and in the USP/NF.
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Routes of administration include, but are not limited to, intramuscular, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraocularly and oral as well as transdermally or by inhalation or suppository. Preferred routes of administration include intravenous, intramuscular, oral, intraperitoneal, intradermal, intraarterial and subcutaneous injection. dsRNAs or dsRNA expression constructs may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microprojectile bombardment gene guns". Alternatively, the dsRNA and/or dsRNA expression construct may be introduced by various means into cells that are removed from the individual. Such means include, for example, *ex vivo* transfection, electroporation, microinjection and microprojectile bombardment. After the gene construct is taken up by the cells, they are reimplanted into the individual. It is contemplated that otherwise non immunogenic cells that have gene constructs incorporated therein can be implanted into the individual even if the host cells were originally taken from another individual.

Routes of administration include, but are not limited to, intramuscular, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraocularly and oral as well as transdermally or by inhalation or suppository. Preferred routes of administration include intravenous, intramuscular, oral, intraperitoneal, intradermal, intraarterial and subcutaneous injection.

Targeted transfection of hepatocytes *in vivo* for delivery of dsRNAs against HBV and/or HCV may be accomplished through IV injection with a composition comprising a DNA or RNA expression vector as described herein complexed with a mixture (e.g., a 35%/65% ratio) of a lactosyl spermine (mono or trilactosylated) and cholesteryl spermine (containing spermine to DNA at a charge ratio of 0.8). Such compositions are especially useful for pharmaceutical applications and may readily be formulated in a suitable sterile, non-pyrogenic vehicle, e.g., buffered saline for injection, for parenteral administration, e.g., IV (including IV infusion), IM, SC, and for intraperitoneal administration, as well as for aerosolized formulations for pulmonary delivery via inhalation. In certain formulations, a DNA expression construct of the invention may be complexed with an endosomolytic spermine such cholesteryl spermine alone, without a targeting spermine; some routes of administration, such as intraperitoneal injection or infusion, may achieve effective hepatic delivery and transfection of a DNA construct of the invention, and

expression of RNA effector molecules, e.g., multiple dsRNA hairpins effective against HBV and/or HCV.

Intraperitoneal administration (e.g., ultrasound guided intraperitoneal injection) of a sterile pharmaceutical composition comprising dsRNA effector molecules and/or dsRNA expression constructs which provide dsRNA effector molecules against HBV and/or HCV in a specially formulated delivery vehicle may be an advantageous route of delivery to promote uptake by liver cells, including hepatocytes. In some compositions the dsRNA expression construct may be complexed with an appropriate transfection enhancing agent, e.g., with a mixture of a lactosyl spermine (mono or trilactosylated) and cholesteryl spermine, or in other compositions with an endosomolytic spermine such cholesteryl spermine alone, without a targeting spermine. The volume, concentration, and formulation of the pharmaceutical composition as well as the dosage regimen may be tailored specifically to maximize cellular delivery while minimizing toxicity such as an inflammatory response. E.g, relatively large volumes (5, 10, 20, 50 ml or more) with corresponding low concentrations of active ingredients, as well as the inclusion of an anti-inflammatory compound such as a corticosteroid, may be utilized if desired. Formulations as known to those of skill in the art of pharmaceutics may also be utilized to provide sustained release of dsRNA effector molecules and/or dsRNA expression constructs of the invention.

dsRNAs or dsRNA expression constructs may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microprojectile bombardment gene guns". Intraperitoneal injection may be accomplished, e.g., with a traditional syringe, with placement of the needle advantageously guided by ultrasound or a similar technique. Alternatively, the dsRNA and/or dsRNA expression construct may be introduced by various means into cells that are removed from the individual. Such means include, for example, ex vivo transfection, electroporation, microinjection and microprojectile bombardment. After the gene construct is taken up by the cells, they are reimplanted into the individual. It is contemplated that otherwise non-immunogenic cells that have gene constructs incorporated therein can be implanted into the individual even if the host cells were originally taken from another individual.

In HBV infected individuals it is anticipated that the dsRNA compositions of the invention may be useful as a pre-treatment in conjunction with therapeutic vaccination protocols

designed to boost immunity against the virus. It is also anticipated that the dsRNA compositions of the invention may be useful for prophylaxis in a regimen of periodic administrations to individuals who because of occupational or other potential for exposure are considered at high risk of exposure to HBV and/or HCV, e.g., fire, emergency, and health care personnel. Such an effective prophylactic regime may include administration of a composition that provides an HBV and/or HCV dsRNA of the invention, e.g., weekly, biweekly, monthly, bimonthly, every three months, every four months, semi-yearly, or yearly, as can be determined through routine experimentation by those of skill in the art of clinical medicine. The ability of a dsRNA expression vector such as a plasmid or viral vector to express the dsRNAs of the invention over a relatively prolonged period of time, expected to be in the range of weeks to months, is considered to be advantageous for this and other applications.

Dosage of dsRNAs For administration of dsRNA (e.g., a short dsRNA to inhibit toxicity or a short or long dsRNA to silence a gene) to an animal, typically between 10 mg to 100 mg, 1 mg to 10 mg, 500 µg to 1 mg, or 5 µg to 500 µg dsRNA is administered to a 90-150 pound person/animal (in order of increasing preference). For administration of a vector encoding dsRNA (e.g., a short dsRNA to inhibit toxicity or a short or long dsRNA to silence a gene) to an animal, typically between 100 mg to 300 mg, 10 mg to 100 mg, 1 mg to 10 mg, 500 µg to 1 mg, or 50 µg to 500 µg dsRNA expression vector or construct is administered to a 90-150 pound person/animal (in order of increasing preference). The dose may be adjusted based on the weight of the animal. In some embodiments, about 1 to 10 mg/kg or about 2 to 2.5 mg/kg is administered. Other doses may also be used, as determined through routine experimentation by those of skill in the art of clinical medicine.

~~For administration in an intact animal, typically between 10 ng and 50 µg, between 50 ng and 100 ng, or between 100 ng and 5 µg of dsRNA or DNA encoding a dsRNA is used. In desirable embodiments, approximately 10 µg of a DNA or 5 µg of dsRNA is administered to the animal. With respect to the methods of the invention, it is not intended that the administration of dsRNA or DNA encoding dsRNA to cells or animals be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration sufficient to provide a dose adequate to inhibit gene expression, prevent a disease, or treat a disease.~~

For administration in an intact animal, e.g., a human subject infected with HBV and/or HCV, between 1 mg and 100 mg, typically between 1 mg and 10 mg, between 10 ng and 50 µg, between 50 ng and 100 ng, or between 100 ng and 5 µg of dsRNA or DNA encoding one or more dsRNA effector molecules is used. In desirable embodiments, approximately 10 µg of a

5 DNA or 5 µg of dsRNA is administered to the animal. In a desirable embodiment, a pharmaceutical composition for parenteral administration is prepared containing 10 mg of a plasmid dsRNA expression construct of the invention (in some formulations complexed with an appropriate transfection facilitating agent such as cholesteryl spermine or a mixture of cholesteryl spermine/trilactosyl spermine) in 25 ml of a suitable sterile vehicle for injection such

10 as Normal Saline Injection, D5W, D5%/0.45% NaCl, D5%/0.2% NaCl, etc., and injected intraperitoneally over 5 to 10 minutes, with needle placement guided by ultrasound or a similar technology. Administration may be repeated periodically, e.g., weekly or monthly, as required. With respect to the methods of the invention, it is not intended that the administration of dsRNA or DNA encoding dsRNA to cells or animals be limited to a particular mode of administration,

15 dosage, or frequency of dosing; the present invention contemplates all modes of administration sufficient to provide a dose adequate to inhibit gene expression, prevent a disease, or treat a disease.

If desired, short dsRNA is delivered before, during, or after the exogenous delivery of dsRNA (e.g., a longer dsRNA) that might otherwise be expected to induce cytotoxicity. See the
20 teaching of USSN 10/425,006, filed 28-Apr-2003, "Methods of Silencing Genes Without Inducing Toxicity", Pachuk.

Therapeutic Advantages of the Invention as it Relates to Worldwide Disease Incidence and Viral Variability

25 The mutability of the hepatitis C virus genome, and to a lesser but significant extent, the hepatitis B virus genome, has been described above as presenting challenges to the design of nucleic acid based therapeutics against these viral agents. The inventors have painstakingly aligned thousands of individual HCV and HBV sequences, originally deriving from thousands of human viral isolates from widely divergent geographic areas worldwide. In doing so, the instant

30 invention has identified and specified preferred sequences which are utilized singly and in

combination in dsRNA effector molecules which target the least mutable regions of the genome of HCV and/or HBV.

The two-fold rationale for this has been discussed above, primarily in terms of ensuring that during the course of infection of a patient with HBV or HCV, the therapeutic of the invention will remain potent against the virus even as it mutates during the course of disease in a given patient. However, the second part of this rationale for deriving and using highly conserved sequences for design of dsRNA-based therapeutic applications, is that this also increases the certainty that the therapeutic dsRNA effector molecules of the invention, particularly the methods and compositions of the invention which utilize combinations of highly conserved sequences in dsRNA effector molecules against HBV and/or HCV, will be able to treat the viral infection present in individuals from a global variety of different ancestries, genetic makeup, and geographical distribution, which are known to manifest in clusters of viral variants based on such factors. Thus, a key feature of the therapeutic utility and novelty of this invention lies in the method of derivation of the preferred sequences and embodiments, and not simply in the demonstration that any particular HCV or HBV dsRNA sequence can inhibit viral replication of one or a few chosen viral isolates (or their cognate replicons) in a laboratory experiment, i.e., in a cell line or animal model, not necessarily reflective of the broad diversity of the HBV and/or HCV virus worldwide, or even in a particular infected individual over the course of infection.

For example, the hepatitis B virus has four subtypes of surface antigen, namely adw, ayw, adr and ayr. While lamivudine is considered an effective therapy for chronic Hepatitis B, a recent study of HBV resistance demonstrated a 20-fold increase in resistance in the adw subtype, compared to the ayw subtype. B Zollner et al. "20-fold Increase in Risk of Lamivudine [Epivir HBV] Resistance in Hepatitis B Virus Subtype adw"; *The Lancet*. 2001; 357: 934-935. In contrast to such conventional antiviral agents, the dsRNA agents of the invention, (e.g., dsRNA effector molecules and expression constructs of the invention, especially when used in combination as taught herein) which utilize HBV and/or HCV sequences highly conserved across such geographical genetic variants are expected to exhibit highly advantageous antiviral activity.

Similarly, HCV is also known for having a wide range of geographically divergent viral genotypes, subtypes, quasispecies, with the following current general global patterns of genotypes and subtypes:

- 1a - mostly found in North & South America; also common in Australia
1 b - mostly found in Europe and Asia.
2a - is the most common genotype 2 in Japan and China.
2b - is the most common genotype 2 in the US and Northern Europe.
5 2c - the most common genotype 2 in Western and Southern Europe.
3a - highly prevalent here in Australia (40% of cases) and South Asia.
4a - highly prevalent in Egypt
4c - highly prevalent in Central Africa
5a - highly prevalent only in South Africa
10 6a - restricted to Hong Kong, Macau and Vietnam
7a and 7b - common in Thailand
8a, 8b & 9a - prevalent in Vietnam
10a & 11a - found in Indonesia
- Accordingly, the highly conserved sequences of the invention, which are expected to be
15 conserved among most if not all of these divergent HCV genotypes and subtypes worldwide,
including 1a, 1b, 2a, 2b, and 2c, are considered highly effective therapeutics agents, e.g., when
utilized as dsRNA effector molecules, especially combinations thereof, and dsRNA expression
vectors capable of expressing such dsRNAs.

Applicants specifically incorporate the entire content of all cited references in this
20 disclosure. Further, when an amount, concentration, or other value or parameter is given as
either a range, preferred range, or a list of upper preferable values and lower preferable values,
this is to be understood as specifically disclosing all ranges formed from any pair of any upper
range limit or preferred value and any lower range limit or preferred value, regardless of whether
ranges are separately disclosed. Where a range of numerical values is recited herein, unless
25 otherwise stated, the range is intended to include the endpoints thereof, and all integers and
fractions within the range. It is not intended that the scope of the invention be limited to the
specific values recited when defining a range.

EXAMPLES

The following Examples are provided as illustrative only. All references mentioned
30 within this disclosure are specifically incorporated herein by reference in their entirety.

EXAMPLE 1Silencing HBV replication and expression in a replication
competent cell culture model

Brief description of cell culture model: A human liver-derived cell line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession V01460. Following internalization into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg (Hepatitis B Surface Antigen) particles into the media of transfected cells. This transfection model reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression and replication.

~~Using this model, cells were co-transfected with the infectious molecular clone of HBV and various eiRNA constructs. The cells were then monitored for loss of HBV expression and replication as described below. Details on the vector and encoded RNAs used in this experiment are provided at the end of this example.~~

Experiment 1:

~~The following is an example of an experiment that was performed using eiRNA vectors encoding sequences derived from GenBank accession number V01460. HBV sequences in these described eiRNA vectors were highly conserved sequences identified as described elsewhere herein, which also exhibited activity as siRNAs (See, Pachuk, C., "Methods and Constructs for Evaluation of RNAi targets and Effector Molecules," PCT/US2004/005065, filed 25 Feb 2004). The particular eiRNA backbone vector used for this experiment was a proprietary vector containing a U6 promoter to drive expression of the encoded RNAs. Each vector encoded only one short hairpin RNA (shRNA). The shRNA coding sequence was followed by an RNA pol III termination sequence. Sequences of the U6 promoter, RNA pol III termination signal, and encoded shRNAs are all shown at the end of the example. Similar vectors containing U6~~

promoters and RNA pol III termination signals are commercially available such as the “siLentGene 2 Cloning Systems” vector from Promega, Inc., Madison, Wis. One of ordinary skill in the art can also create them according to the information provided herein. It is expected that similar results would also be obtained using other expression and promoter systems especially those vectors with RNA pol III promoters that are not U6, for example H1 promoters or 7SK promoters.

Using this model, cells were co-transfected with the infectious molecular clone of HBV and various eiRNA constructs (dsRNA expression constructs). The cells were then monitored for loss of HBV expression and replication as described below. Details on the vector and encoded RNAs used in this experiment are provided at the end of this example.

Experiment 1:

The following is an example of an experiment that was performed using eiRNA vectors (dsRNA expression vectors) encoding sequences derived from GenBank accession number V01460. HBV sequences in these described eiRNA vectors were highly conserved sequences identified as described elsewhere herein, which also exhibited activity as siRNAs (See, Pachuk, C., “Methods and Constructs for Evaluation of RNAi targets and Effector Molecules,” PCT/US2004/005065, filed 25-Feb-2004). The particular eiRNA backbone vector used for this experiment was a proprietary vector containing a U6 promoter to drive expression of the encoded RNAs. Each vector encoded only one short hairpin RNA (shRNA). The shRNA coding sequence was followed by an RNA pol III termination sequence. Sequences of the U6 promoter, RNA pol In termination signal, and encoded shRNAs are all shown at the end of the example. Similar vectors containing U6 promoters and RNA pol III termination signals are commercially available such as the “siLentGene-2 Cloning Systems” vector from Promega, Inc., Madison, Wis. One of ordinary skill in the art can also create them according to the information provided herein. It is expected that similar results would also be obtained using other expression and promoter systems especially those vectors with RNA pol III promoters that are not U6, for example H1 promoters or 7SK promoters.

Experimental Procedure: Transfection.

Huh7 cells cultured in RPMI-1640 media were seeded into six-well plates at a density of 3×10^5 cells/well. All transfections were performed the day after cell seeding using Lipofectamine™ (InVitrogen, Carlsbad, Cal.) according to the manufacturer's directions. In this experiment, cells were transfected with 500 ng of the infectious HBV plasmid ayw subtype ("pHBV2") (GenBank Accession # V01460) and 500 ng, 300ng, 250ng, 120 ng, 100 ng, 50ng, or 10 ng of an eiRNA construct. DNA was held constant/transfection at 2.5 μ g by including an inert plasmid DNA, pGL3-Basic (Promega, Madison Wis.) in amounts that brought the total DNA in the transfection to 2.5 μ g. For example, in transfections receiving 500 ng of HBV DNA and 500 ng of an eiRNA construct, 1.5 μ g pGL3 was added to the transfection. Prior to transfection, media was removed from the cells and the cells washed with Opti-MEM® (InVitrogen Life Technologies, Carlsbad, Cal.). 800 μ l of Opti-MEM® was then added to each well of cells followed by the addition of the transfection mix. Seventeen to nineteen hours post-transfection, the transfection mix and Opti-MEM® were removed from cells and replaced with 2 mL culture media/well. At 3, 6, and 10 days after transfection, the media was removed from cells and stored at -70 °C. The media was replaced with 2 mL of fresh culture media on days 3 and 6. All transfections were carried out in duplicate. Two sets of control transfections were also performed: HBV DNA alone (500 ng HBV DNA plus 2 μ g pGL3) and HBV DNA with a control eiRNA construct (500 ng HBV DNA, 1 μ g control eiRNA construct, and 1.0 μ g pGL3 DNA).

Monitoring cells for loss of HBV expression.

Following transfection, cells were monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion. Cells were monitored by assaying the media of transfected cells (and a media control) at days 3, 6, and 10 post-transfection. The Auszyme® ELISA, commercially available from Abbott Labs (Abbott Park, Ill.), was used to detect surface Ag (sAg) according to the manufacturer's instructions. sAg was measured since surface Ag is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected infectious HBV clone and from HBVcccDNA produced during infection *in vivo*. Since surface Ag synthesis can continue with deleterious

effects in the absence of HBV replication, it is important to down-regulate not only viral replication but also replication-independent synthesis of sAg.

Results:

Cells transfected with the HBV-specific eiRNA constructs described at the end of this example all induced a decrease in sAg levels relative to the controls. The level inhibition is shown in the accompanying FIG. 2-8 corresponding to data found in Tables 2-8. Note that the sequences identified as 788-808 and 807-827 only lowered surface Ag levels by 30% and 50% respectively at 500 ng doses. These are the only two eiRNAs that do not target the sAg mRNA; instead they target the 3.1 Kb HBV mRNAs and therefore reduce sAg levels indirectly. The 30% to 50% reduction in sAg observed when these other HBV RNAs are targeted is considered a strong indication that these eiRNA constructs are efficacious.

HBV-specific eiRNAs used in this experiment

The eiRNA vectors encode the HBV sequences listed in Table 1. The sequences are shown as well as their map coordinates on GenBank accession number V01460. At the rightmost part of the table is the SEQ ID NO that these sequences map within. The sequence of the encoded RNA is 5'GGTCGAC (a sequence that is *per se* unimportant, but is derived from the polylinker sequence of the particular vector used) followed by a first sense or antisense HBV sequence followed by the loop sequence (underlined in Table 1) followed by a second HBV sequence, which is the complement to the first HBV sequence. Note that the loop structure does not need to be a fixed sequence or length, and we have used several loop sequences with no significant impact on the functioning of the eiRNA construct. The second HBV sequence is followed by a string of T residues, e.g., 1, 2, 3, or more Ts, that function as the termination signal for RNA pol III.

Table 1

<u>HBV AYW</u>	<u>SEQ ID</u>	<u>sequence (sense stem – loop antisense stem)</u>	<u>Maps within</u>
<u>coordinates*</u>	<u>NO</u>		<u>SEQ ID NO</u>
<u>788-808</u>	<u>14</u>	<u>CGTCTGGAGGGAGGGAGTTAGAGAACTTAAC</u> <u>TCCCTCGCCTCGCAGACG</u>	<u>5, 6, 7, or 8</u>
<u>807-827</u>	<u>15</u>	<u>TTCTTCTCTAGGGGACCTGCAGAGAACTTG</u> <u>CAGGTCCCCTAGAAGAAGAA</u>	<u>5, 6, 7, or 8</u>
<u>1291-1311</u>	<u>16</u>	<u>AAGCCACCCAAGGCACAGCTTAGAGAACTT</u> <u>AAGCTGTGCCCTGGTGGCTT</u>	<u>4</u>
<u>1299-1319</u>	<u>17</u>	<u>CAAGGCACAGCTGGAGGCTTAGAGAACTT</u> <u>AAGCCTCCAGCTGTGCCCTG</u>	<u>4</u>
<u>1737-1757</u>	<u>18</u>	<u>GGATTCAAGCGCCGACGGGACGAGAGAA</u> <u>CTTCGTCGGATCCATACTGGGAA</u>	<u>10</u>
<u>1907-1927</u>	<u>19</u>	<u>TTCCGCAGTATGGATCGGCAGAGAGAA</u> <u>CTTCTGCCGATCCATACTGGGAA</u>	<u>3</u>
<u>1912-1932</u>	<u>20</u>	<u>CAGTATGGATCGGCAGAGGAGAGAGAA</u> <u>ACTTCTCCCTGCCCATCCATACTG</u>	<u>3</u>
<u>1943-1963</u>	<u>21</u>	<u>TCCACGCATGCGCTGATGGCCAGAGAA</u> <u>ACTTGGCCATCAGCGCATGGTGG</u>	<u>3</u>
<u>1991-2011</u>	<u>22</u>	<u>TGGTCAGCAAACACTTGGCAAGAGAA</u> <u>ACTTTGCCAAGTGTGCTGACGCA</u>	<u>3</u>
<u>2791-2811</u>	<u>23</u>	<u>AAAACGCCGAGACACATCCAAGAGAA</u> <u>ACTTTGGATGTGTGCCGCGTTT</u>	<u>2</u>
<u>2791-2811mut</u>	<u>24</u>	<u>AAAACACCACACACGCATCCAAGAGAA</u> <u>ACTTTGGATGCCGTGTGGTGT</u>	<u>2</u>
<u>2912-2932</u>	<u>25</u>	<u>TTGAGAGAAAGTCCACCAAGGAGAGAA</u> <u>ACTTCTCGTGGTGGACTTCTCTCAA</u>	<u>1</u>
<u>2919-2939</u>	<u>26</u>	<u>AAGTCCACCAACGAGTCTAGACAGAGAA</u> <u>ACTTGTCTAGACTCGTGGTGGACTT</u>	<u>1</u>

*nucleotide coordinate refer to Genbank accession number V01460.

Table 1

<u>HBV-AYW</u> <u>coordinates*</u>	<u>SEQ ID</u> <u>NO</u>		<u>Maps within</u> <u>SEQ ID NO</u>
<u>788-708</u>	14	CGTCTGCGAGGCAGGGAGTTAGAGAA <u>ACTTA</u> ACTCCCTGCCCTCGCAGACG	5, 6, 7, or 8
<u>807-827</u>	15	TTCTTCTTAGGGGACCTGCAGAGAA <u>ACTTG</u> CAGGTCCC <u>TAGA</u> AAGAAGAA	5, 6, 7, or 8
<u>1291-1311</u>	16	AAGCCACCCAAGGCACAG <u>CTTAGAGAA</u> ACTTAAG <u>GCTGTGCCTGGTGGCTT</u>	4
<u>1299-1319</u>	17	CAAGGCACAG <u>CTGGAGGCTT</u> AGAGAA <u>ACTTAAGCCTCCAAG</u> GCTGTGCCTG	4
<u>1737-1757</u>	18	GGATT <u>CAGCGCCGACGGACGAGAGAA</u> CTTCG <u>TCGGCTGAATCC</u>	10
<u>1907-1927</u>	19	TTCCGCAGTATGGAT <u>CGGCAGAGAGAA</u> CTTCTGCC <u>ATCCATACTGCGGAA</u>	3
<u>1912-1932</u>	20	CAGTATGGAT <u>CGGCAGAGGAGAGAGAA</u> CTTCTCC <u>CTGCCATCCATACTG</u>	3
<u>1943-1963</u>	21	TCCACGCAT <u>CGCCTGATGGCCAGAGAA</u> ACTTG <u>GCCATCGCGATGCGTGGAA</u>	3
<u>1991-2011</u>	22	TGCGTCAGCAA <u>ACACTTGGCAAGAGAA</u> CTTG <u>CCAAGTGTGCTGACGCA</u>	3
<u>2791-2811</u>	23	AAAACGCCGCAGACACAT <u>CCAAGAGAA</u> CTTGG <u>ATGTGCTGCCGTTT</u>	2
<u>2791-2811mut</u>	24	AAAACACCACAC <u>CGCATCCAAGAGAA</u> CTTGG <u>ATGCGTGTGGTTT</u>	2
<u>2912-2932</u>	25	TTGAGAGAA <u>GTCCACCACGAGAGAGAA</u> CTTCT <u>CGTGGACTCTCTCAA</u>	1
<u>2919-2939</u>	26	AAGTCCACCACGAGT <u>CTAGACAGAGAA</u> CTTGT <u>CTAGACTCGTGGACTT</u>	1
<u>799-779</u>	49	GCCTCGCAGACGAAGGT <u>CTCAAGAGAA</u> CTT <u>GAGACCTCGTCTCGAGGC</u>	

* nucleotide coordinates refer to Genbank accession number V01460.

A diagram of the transcribed RNA structure is shown in **FIG. 9**.

SEQ ID NO:13 is the nucleotide sequence of U6 promoter. Nucleotide sequence of RNA pol III terminator: 5' – TTTTT – 3'.

The HBV sequitopes of Table 1 (without their respective complement sequence and
 5 without the “loop” or linker sequence utilized in a “hairpin” or stem-loop dsRNA effector
molecule) are shown in Table 1A below. Each such HBV sequitope, together with its
complementary sequence, optionally with an appropriate loop or linker sequence as taught
herein, may be utilized in a dsRNA effector molecule of the invention (e.g., a duplex dsRNA or
hairpin dsRNA effector molecule, or encoded within a dsRNA expression vector). In one aspect,
 10 multiple (e.g., 2, 3, 4, 5, 6, or more) of such dsRNA short hairpin effector molecules are encoded
in a single expression vector, e.g., a plasmid expression vector, each under the control of a
different promoter, e.g., a polymerase III promoter, as described elsewhere herein.

TABLE 1A

<u>HBV-AYW coordinates</u> <small><u>Genbank accession number V01460*</u></small>	<u>SEQ ID NO</u>	<u>Conserved HBV Sequitope</u>	<u>Maps within SEQ ID NO</u>
<u>788-808</u>	<u>50</u>	<u>CGTCTGCGAGGCGAGGGAGTT</u>	<u>5, 6, 7, or 8</u>
<u>807-827</u>	<u>51</u>	<u>TTCTTCTTCTAGGGGACCTGC</u>	<u>5, 6, 7, or 8</u>
<u>1291-1311</u>	<u>52</u>	<u>AAGCCACCCAAGGCACAGCTT</u>	<u>4</u>
<u>1299-1319</u>	<u>53</u>	<u>CAAGGCACAGCTTGGAGGCTT</u>	<u>4</u>
<u>1737-1757</u>	<u>54</u>	<u>GGATTCA CGGCCGACGGGACG</u>	<u>10</u>
<u>1907-1927</u>	<u>55</u>	<u>TTCCCGCAGTATGGATCGGCAG</u>	<u>3</u>
<u>1912-1932</u>	<u>56</u>	<u>CAGTATGGATCGGCAGAGGAG</u>	<u>3</u>
<u>1943-1963</u>	<u>57</u>	<u>TCCACGCATCGCCTGATGGCC</u>	<u>3</u>
<u>1991-2011</u>	<u>58</u>	<u>TGCGTCAGCAAACACTTGGCA</u>	<u>3</u>
<u>2791-2811</u>	<u>59</u>	<u>AAAACGCCGCAGACACATCCA</u>	<u>2</u>
<u>2912-2932</u>	<u>60</u>	<u>TTGAGAGAA GTCCACCACGAG</u>	<u>1</u>
<u>2919-2939</u>	<u>61</u>	<u>AAGTCCACCACGAGTCTAGAC</u>	<u>1</u>
<u>799-779</u>	<u>62</u>	<u>GCCTCGCAGACGAAGGTCTCA</u>	

15

Tables and Graphs.

HBsAg was measured as described above and plotted in **FIG. 2-8** corresponding to the data in Tables 2-8. The amount of eiRNA construct is shown in parentheses following the name

of the eiRNA construct and is in μg amounts. For example, 2791(0.5) means that 0.5 μg or 500 ng of eiRNA construct 2791-2811 (see Table 1) was used in the transfection. The percent inhibition relative to the control is also shown in the tables below and it is specific for the day 10 measurement. Note that the 4th set of data in this example in which 1299 was evaluated at 500 ng has only two timepoints, days 3 and 6, because the evaluation was not carried out at day 10. The percent inhibition for this experiment was shown for day 6 data. Data is shown as raw OD 5 data collected as described by the manufacturer of the Auszyme ELISA assay kit used to measure sAg. Not shown are the 50 ng data for 2791-2811 and the 10ng data for 1907-1927. Each of these doses inhibited HBsAg expression by about 50% relative to the control.

10 Table 2

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.339	1.88	3.268	-----
2791(0.5)	0.101	0.263	0.333	89.8

Table 3

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	1.169	4.445	10.18	-----
2791(0.5)	0.442	0.743	1.3	87.2
2791Mut(0.5)	1.136	4.305	10.595	-----

15

Table 4

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.375	1.952	4.005	-----
2791mut(1)	0.421	1.847	4.753	-----
HCV(1)	0.445	1.805	3.933	-----
788(0.5)	0.255	1.195	2.778	30.6
807(0.5)	0.254	1.326	2.015	49.7
1907(0.25)	0.052	0.113	0.365	90.9
1912(0.25)	0.138	0.208	0.517	87.1
1943(0.25)	0.099	0.233	0.506	87.4
1991(0.25)	0.075	0.152	0.291	92.7
2912(0.25)	0.095	0.183	0.331	91.7

Table 5

5

	Day 3	Day 6	% Inhibition relative to control
pHBV2	0.474	1.513	-----
1299(0.5)	0.439	0.699	53.8

Table 6

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.33	1.617	2.88	-----
2791(0.3)	0.103	0.192	0.349	87.9
1737(0.3)	0.051	0.094	0.232	91.9
1291(0.12)	0.239	0.587	1.195	58.5
1907(0.12)	0.043	0.086	0.356	87.6
2919(0.12)	0.218	0.565	1.09	62.2

Table 7

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.741	2.53	5.383	-----
2791(0.3)	0.223	0.256	0.458	91.5
1737(0.1)	0.212	0.351	0.549	89.8
1907(0.1)	0.067	0.149	0.468	91.3
1991(0.1)	0.067	0.16	0.345	93.6

Table 8

5

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.864	4.414	8.344	-----
1907(0.05)	0.17	0.538	1.396	83.3
2919(0.1)	0.368	1.044	1.908	77.1
1291(0.2)	0.573	1.654	1.896	77.3

Experiment 2:

Background: The same cell culture model was used to evaluate the additive effects of adding two eiRNA constructs. In this experiment 2791-2811 and 2919-2939 were evaluated.

- 10 They were evaluated separately at two doses: 10 ng and 25 ng, and in combination at 10 ng (5 ng of 2791-2811 plus 5 ng of 2919-2939) and at 25 ng (12.5 ng 2791-2811 plus 12.5ng 2919-2939). An additive effect is observed, for example, when half the inhibition seen with 25 ng 2791-2811 plus half the inhibition seen with 25 ng 2919-2939 is about equal to the inhibition seen of the 25 ng combination dose. This is important because while one may not be gaining inhibition over
 15 the use of a single eiRNA construct at the 25 ng dose, the use of two or more eiRNA sequences is very important in preventing the generation of viral escape mutants.

Experimental Procedure: Transfection.

Huh7 cells were seeded into six-well plates at a density of 3×10^5 cells/well. All transfections were performed the day after cell seeding using Lipofectamine™ (InVitrogen)
 20 according to the manufacturer's directions. In this experiment, cells were transfected with 500 ng of the infectious HBV plasmid ayw subtype (GenBank Accession # V01460) and 25 ng or 10 ng of two separate eiRNA constructs or a combination of these two eiRNA constructs at a total

of 25 ng or 10 ng. DNA was held constant/transfection at 2.5 µg by including an inert plasmid DNA, pGL3, in amounts that brought the total DNA in the transfection to 2.5 µg. For example, in transfections receiving 500 ng of HBV DNA and 10 ng of an eiRNA construct, then 1.99 µg pLUC was added to the transfection. Prior to transfection, media was removed from the cells
 5 and the cells washed with Opti-MEM® (InVitrogen Life Technologies). 800 µl of Opti-MEM® was then added to each well of cells followed by the addition of the transfection mix. Seventeen to nineteen hours post-transfection, the transfection mix and Opti-MEM® was removed from cells and replaced with 2 mL culture media/well. At 4, 8, and 11 days after transfection, the media was removed from cells and stored at -70 °C. The media was replaced with 2 mL of fresh
 10 culture media on days 4 and 8. All transfections were carried out in duplicate. Two sets of control transfections were also performed: HBV DNA alone (500 ng HBV DNA plus 2 µg pGL3), and HBV DNA with a control eiRNA construct (500 ng HBV DNA, 500 ng control eiRNA construct and 1.5µg pGL3. DNA).

Results:

15 Results are shown in Table 9, and the corresponding graph found in **FIG. 10**. Combining 2791-2811 and 2919-2939 showed at least equal effects to administration of 2791-2811 or 2919-2939 alone. It is expected that similar advantages will be seen by combining two or more dsRNAs directed to different HBV sequences from the same and/or different HBV genes.

20 Table 9

	Day 4	Day 8
pHBV ₂	3.74	15.03
2791 @ 25 ng	2.49	9.63
2919 @ 25 ng	2.55	10.07
2791 + 2919 @ 25 ng	2.73	10.91

Experiments 3 and 4

Silencing of HBV in a mouse model.

Summary: Two of the eiRNA vectors described in confirmatory experiment 1 were
 25 assessed for their ability to silence an HBV replicon in a mouse model. These vectors were the 2791-2811 and the 1907-1927 vectors. Both vectors were found to silence HBV in the mouse model to a similar extent as they silenced in the cell culture model. The ability to silence this

HBV replicon in mice by other therapeutics has been demonstrated to be a predictor of human efficacy [4].

Animal Model Background:

Chimpanzees represent the only animal model in which to study human HBV infectivity.

- 5 A mouse model is available, however, in which HBV expression and replication occur. This model has been invaluable for the evaluation of anti-HBV therapeutic agents not only targeted against viral replication but also against RT-independent expression of antigen. In this model, replication competent HBV is expressed transiently from episomal HBV DNA. This model is created by introducing replication competent HBV DNA into mouse liver by hydrodynamic
10 delivery [1].

The aim of the following experiment was to test two of the vectors encoding HBV-specific sequences evaluated in Experiment 1 for efficacy in a mouse model even though there were not expected to be HBV-sequence-related efficiency differences between the cell culture and mouse models. This experiment utilized hydrodynamic delivery as a method to co-deliver
15 replication competent HBVayw plasmid (Example 1, confirmatory experiment 1) with an effector HBV-specific eiRNA expression vector. Hydrodynamic delivery is ideal for these first studies because it results in efficient delivery of nucleic acid to the liver [5].

Experiments.

Hydrodynamic Delivery Studies: Experiment 3.

- 20 All animals were hydrodynamically injected with 7.5 pg infectious HBVayw plasmid (described in confirmatory Example 1). Following internalization into hepatocytes and nuclear localization, transcription of HBVayw plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication [1]. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse
25 transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the sera of injected animals. Experimental animals were co-injected with 10 µg 2791-2811. A second group of control animals were injected with 10 pg of an irrelevant eiRNA construct. All animals were also co-injected with 2.5 µg of a GFP reporter plasmid (Clontech, Palo Alto, Cal.). Expression of GFP mRNA in the livers of injected mice served as a control to normalize
30 results against the mouse model transfection efficiency. Total DNA injected in animals was kept

at a constant 20 µg by including pGL3, an inert filler DNA (Promega, Madison, Wis.). All DNA was formulated and injected according to the methods described in Yang et al. [1]. There were 5 animals per group. The DNAs and amounts of DNA injected per animal are shown in Table 10.

5 Table 10

Group	HBV DNA	GFP DNA	eiRNA	pGL3
1	7.5 µg	2.5 µg	10 µg 2791	0 µg
2	7.5 µg	2.5 µg	10 µg control	0 µg
3	7.5 µg	2.5 µg	0 µg	10 µg

Timepoints of analysis were selected based on published results from Dr. Chisari's laboratory [1], which detail the kinetics of HBVayw plasmid replication in mice following hydrodynamic delivery. Serum was assayed for the presence of HBsAg on days 1, 2, 3, and 4
10 post-injection. Assays were performed as described for the cell culture model of HBV replication. The presence of HBV RNA in liver samples was ascertained by Northern blot analysis on day 2 following injection using procedures developed in Dr. Chisari's laboratory [1] and normalized to endogenous GAPDH RNA levels and GFP mRNA levels using conventional techniques, or a quantitative RT-PCR assay for HBV RNAs containing sAg coding sequences
15 using standard techniques. RT-PCR is more quantitative than Northern Blot analysis and has a larger dynamic window than does Northern Blot analysis.

Downregulation of both HBV RNA by Northern Blot analysis and HBsAg were seen in mice injected with 2791-2811. See **FIG. 11**. Also not shown, quantitative RT-PCR demonstrated the presence of 867 HBV RNA molecules in the livers of control mice and 57
20 molecules of HBV RNA in 2791-2811 treated mice, a 15-fold downregulation.

Table 11

Group	Mouse	3.5 kb	2.1 kb	GFP	HBV total	HBV/GFP	Group Average	Std. Dev
10 µg 2791	1	182360	1440614	4044344	1622974	4.0	3.0	0.76
	2	392294	3161703	9954889	3553997	3.6		
	3	268673	3114347	15317275	3383020	2.2		
	4	394799	3909096	16806285	4303895	2.6		
	5	362182	4439430	18306755	4801612	2.6		
HBV only	21	2412562	8720964	3860082	11133526	28.8	17.4	7.40
	22	2170741	7958388	6110744	10129129	16.6		
	23	2713213	12060855	9633404	14774068	15.3		
	24	1924373	7243024	11042915	9167397	8.3		
	25	1464641	5726217	3968243	7190858	18.1		

Table 12

NUC5_HBsAg		HBsAG (ng/ml)			
		d1	d2	d3	d4
HBV	2	2810	6793	8422	8517
	3	2344	8332	8089	8743
	4	1684	8788	9064	8876
	5	2318	9378	8597	8480
	29	1066	5038	5153	5925
grp ave=>		2044	7666	7865	8108
Std Dev=>		678	1754	1556	1231
eiHCV	6	2554	8048	9233	8870
	9	2267	8420	9535	8338
	10	1704	8258	8761	7840
	30	1362	4171	5406	4920
	grp ave=>	1972	7224	8234	7492
Std Dev=>		538	2041	1912	1765
2791	11	1262	2823	2276	2080
	12	1222	2549	2858	1593
	14	1056	1933	1143	792
	15	1275	8320	1920	2068
	27	779	4771	3782	1252
grp ave=>		1119	4079	2396	1557
Std Dev=>		209	2598	993	551

5

Hydrodynamic Delivery Studies: Experiment 4.

This experiment was similar to the Experiment 3 of Example 1 except that two eiRNA constructs were evaluated: 2791-2811 and 1907-1927. In this experiment, HBsAg was measured on days 1 and 4 using the assay already described herein.

10

Table 13

NUC6_HBsAg		HBsAG (ng/ml)	
		d1	d4
HBV	2	6147 ^	36,953 ^
	3	6234 ^	42,542 ^
	4	4658	33,061 ^
	5	5077 ^	29,389 ^
	grp ave=>	5529	35486
Std Dev=>		784	5627
eiHCV	6	1901	11,236
	7	6286 ^	29,637 ^
	8	1023	6,345
	grp ave=>	3070	15739
	Std Dev=>	2820	12282
2791	11	3966	5009
	13	4705	7347 ^
	14	2289	4538
	15	2427	4217
	grp ave=>	3347	5278
Std Dev=>		1182	1417
1907	16	4954	7203 ^
	18	2982	6917 ^
	19	3436	7568 ^
	20	2246	5135 ^
	grp ave=>	3405	6706
Std Dev=>		1143	1081

5

A Four-Promoter RNA polymerase III-based expression construct for production of shRNAs which reduce Hepatitis B RNA production and replication.

10 As described in more detail in PCT/US05/29976 filed 23-August-2005, a plasmid expression vector, pHB4, containing 4 polymerase III promoter short hairpin dsRNA expression cassettes was constructed. Each expression cassette included a polymerase III promoter operably linked to a sequence encoding a short hairpin dsRNA, and a polymerase III termination sequence. The polymerase III promoters were U6, 7SK, and two copies of a 7SK sequence
15 variant promoter, designated 7SK-4A. Each short hairpin dsRNA sequence comprised a double-

stranded stem region homologous and complementary to a highly conserved HBV sequence as taught herein. The four dsRNA effector molecules comprised the sequences of SEQ ID NO: 49; SEQ ID NO: 23; SEQ ID NO:19; and SEQ ID NO: 18; which comprise, respectively; the sequences of SEQ ID NO: 62; SEQ ID NO: 59; SEQ ID NO:55; and SEQ ID NO: 54. As described in more detail in Example 1 of PCT/US05/29976, however, the sequence encoding the dsRNA hairpin effector molecule was inserted into an expression cassette of the plasmid expression vector at a restriction site which in effect resulted in several additional nucleotides being added to the 5' end of the ultimate transcript. The predicted transcript which includes the dsRNA hairpin actually contains additional 5' and 3' sequences: a 5' leader consisting of 6 bases (e.g., the Sal I or Hind III or other chosen recognition sequence), followed by the dsRNA hairpin sequences, followed by a short 3' terminal U tract, usually two (1, 2, 3, or 4) U residues incorporated during transcription termination. These differences in length and composition of 5' and 3' transcript sequences flanking the dsRNA hairpin did not appear to adversely affect the ability of the dsRNA hairpin to effect dsRNA-mediated silencing, which suggests that, unlike synthetic dsRNA duplexes, endogenously expressed dsRNA hairpin constructs are effective despite varying in a number of respects, e.g., length of dsRNA "stem" between about 19-29 bp, length and composition of single-stranded loop, presence or absence of additional short 5' and/or 3' sequences.

A luciferase assay as taught in Example 1 of PCT/US05/29976 and in WO 04/076629, published 10-Sept-2004, "Methods and Constructs for Evaluation of RNAi Targets and Effector Molecules" indicated that all 4 promoter/shRNA cassettes were active in silencing their target sequences in a cell supplied with the vector and an assayable substrate. The IC₅₀ value decreased substantially when increasing from a one promoter/shRNA cassette vector to the pHB4 expression vector containing 4 promoter/shRNA cassettes. The 10₅₀ values may have also been affected by the relative potency and transcription levels of each shRNA molecule, and did not reflect a simple relationship to the concentration of the vector only, which in effect behaved as four drugs after entering the cell and expressing the four encoded dsRNA molecules. In other words, the increased potency reflected not only the greater number of total shRNA transcripts generated by the vector, but the also the individual potency that each shRNA has to effect the reduction of sAg or eAg production via degradation of the target viral RNA molecules. The progressive addition of shRNA cassettes increased the potency of the vector in an apparently

quantitative manner, and furthermore increased the pharmacological activity against the HBV target by inhibiting four distinct sites of the HBV target. It is important to recognize, however, that the ability of the multiple polymerase III expression constructs to express multiple individual antiviral dsRNA hairpin molecules is of significant value in and of itself, not just

5 because of associated increases in “potency”. Where the level of antiviral efficacy is high, the incremental quantitative increase in viral inhibition seen with each additional dsRNA molecule may be less important per se than the ability of the constructs to deliver what is in effect a multi-drug regimen, with the inherent advantage of being highly resistant to the development of viral resistance.

10 The expression vectors, designed to deliver multiple dsRNA effector molecules targeting highly conserved HBV and/or HCV polynucleotide sequences, when delivered to a virally infected cell, have the unique ability to destroy the viral nucleic acid products directly. Moreover, inherent and integral to the design and intent of these multiple promoter vectors (which express a plurality of different inhibitory short hairpin dsRNAs targeting different

15 portions of the viral genome), is the property of generating multiple different viral antagonists simultaneously. The antagonists (short hairpin dsRNA effector molecules) target different genome sequences in the viral genome. One of these antagonists would probably be sufficient to disable the virus; however, the redundancy serves as a “backup” mechanism such that if the viral sequence mutates to render one antagonist inert, there are 2, 3, 4 or more additional antagonists

20 available. Additionally, by targeting multiple sites in the viral genome, different DNA or RNA products of the virus which play different roles in the disease pathology can be attacked at the same time.

25 In the case of Hepatitis B for example, in one embodiment, the instant invention uses 4, 5, or more shRNA molecules selected from the following sequences and other highly conserved HBV sequences as taught herein: e.g., “799” (SEQ ID No. 49); “1907” (SEQ ID No. 19); “2791” (SEQ ID No. 23); “1737” (SEQ ID No. 18), “1991” (SEQ ID No. 22), “1943” (SEQ ID No. 21). Other of the conserved HBV sequences disclosed herein, including sequences of e.g., 19 to 29 nucleotides, which comprise all or part of “799”, “1907”, “2791”, “1737”, “1991”, or “1943”, may be selected for inclusion in dsRNA hairpin effector molecules to be expressed by

30 expression vectors comprising multiple promoters, including multiple polymerase III expression vectors. Due to the nature of HBV gene expression and overlapping transcriptional products this

allows targeting of multiple RNA transcripts as well as the replicative template of the virus which will interfere with replication and expression of more than one of the viral proteins. One of the shRNA molecules, “1737” (SEQ ID No. 18) uniquely can disable the RNA encoding a product known as the X protein (HbX). Strong evidence exists in the biomedical literature that

5 the X protein plays a role in establishment and/or maintenance of liver cancer. Because the existing drugs that can to some extent inhibit viral replication cannot eliminate the cell of integrated or other residual copies or portions of the viral genome, these drugs cannot shut off the production of HbX, even in patients “cured” of infectious HBV, and thus can not directly reduce any incidence of cancer that is mediated by dormant HbX. Multiple anti-HBV dsRNA

10 hairpin expression constructs of the present invention can attack both the replication of the virus and the expression of all viral proteins, some which cause the inflammatory insult which results in hepatitis, and others such as HbX, which are believed to promote hepatocellular carcinoma via a distinct but not fully understood mechanism. It is recognized that the principles taught herein can be used to design constructs of the invention specifically tailored to treat such “post

15 infection” patients, which express dsRNAs against Hbx and any other residual HBV antigens.

While the HBV target sequences of the invention were chosen expressly to represent those highly conserved or identical among a large number of different isolates (strains) of HBV, for reference purposes the identified sequences, e.g., shRNA sequences, can be mapped back to HBV isolate AYW. It should be recognized, therefore, that the dsRNAs and dsRNA expression

20 constructs of the invention are expected to be effective not only against HBV AYW and related viral strains, but against nearly all if not all HBV strains encountered in infected human populations in widely dispersed geographical areas.

EXAMPLE 2

25 Hepatitis C- Sequences for RNAi Therapeutic Development

Experiment 1

Brief Introduction:

The hepatitis C virus (HCV) is the primary cause of non-A, non-B transfusion-associated hepatitis and accounts for more than 200 million hepatitis cases worldwide. The HCV genome

30 has a high degree of sequence variability. There are six major genotypes comprising more than fifty subtypes and significant heterogeneity hallmark by quasi-species has been found within

patients. Great progress in understanding HCV replication has been made by using recombinant polymerases or cell-based subgenomic replicon systems. By using a replicon cell system, HCV-specific siRNA has been demonstrated to be able to suppress HCV protein expression and RNA replication. Sequences of the 5' NTR and both structural and nonstructural genes have been
5 targeted successfully. The highly conserved nature of the 3' NTR sequence makes it a highly attractive target for siRNA based therapy. However, no study has been done to examine the feasibility of using the 3' NTR. Here we report the design and testing of several siRNAs that can inhibit HCV protein expression in the subgenomic replicon system. Exogenously synthesized HCV-specific siRNAs were transfected into the HCV replicon cell line as described below.

10 **Cell culture and media:**

The HCV replicon in hepatoma Huh7 cells was cultured in Dulbecco's Modified Eagle Media ("DMEM") (Invitrogen) containing 10% fetal calf serum (Invitrogen), 1% penicillin-streptomycin, 1% non-essential amino acids and 0.5 mg/mL Geneticin. Cells were grown to 75% confluence prior to splitting.

15 **Western blot analysis:**

Total cell lysates from replicon cells were harvested from replicon cells in 1 x LDS Buffer (Invitrogen). The lysates were heated at 90 °C for 5 min in the presence of beta-mercaptoethanol before electrophoresis on a 10% Tris-Glycine polyacrylamide gel (Invitrogen). The protein was transferred to PVDF (Invitrogen) membrane. Following the transfer, the
20 membrane was rinsed once with PBS containing 0.5% Tween-20 (PBS Tween) and blocked in PBS-Tween containing 5% non-fat milk for 1 hr. After washing with PBS-Tween, the membrane was incubated with the primary α -NS5A antibody (a gift from Dr. Chen Liu) at 1:1500 dilution for 1 hr at room temperature. Prior to incubation with HRP conjugated a-mouse IgG secondary antibody (Amersham) diluted 1:5000, the blot was washed in PBS-Tween 20.
25 Following the secondary antibody incubation, the blot was washed again and treated with ECL (Amersham) according to the manufacturer's protocol.

Northern blot:

Total cellular RNA was extracted by using the Rneasy® kit (Qiagen). Northern blot analysis was done according to the protocol of Guo et al. Briefly, 5 μ g total RNA was

electrophoresed through a 1.0% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane and immobilized by UV cross-linking (Stratagene). Hybridization was carried out using α -[³²P]CTP-labeled neomycin RNA in a solution containing 50% deionized formamide, 5x SSC (750 mM sodium chloride, 750 mM sodium citrate), Denhardt's solution,
 5 0.02 M sodium phosphate (pH 6.8), 0.2% sodium dodecyl sulfate ("SDS"), 100 μ g of sheared denatured salmon sperm DNA/ml, and 100 pg of yeast RNA/ml, for 16 hr at 58 °C. The membranes were washed once in 2x SSC/0.1% SDS for 30 min at room temperature and twice in 0.1x SSC/0.1% SDS for 30 min at 68 °C. Membranes were exposed to X-ray film.

Transfection of siRNA into replicon cells:

10 For transfection of siRNA into replicon cells the Lipofectamine® 2000 reagent (Invitrogen) was used according to the user manual. Briefly, 2x10⁴ cells in 0.5 mL of DMEM was seeded in 24 well plates one day before the transfection. The indicated amount of siRNA was diluted in 50 μ L OptiMEM and mixed with diluted Lipofectamine® 2000 reagent (1 μ L in 50 μ L of Optimem). The mixture was incubated at room temperature for 20 min before being
 15 applied onto the cell monolayer. 48-72 hr after transfection, cells were washed in PBS and lysed in 100 μ L. SDS sample buffer.

Table 14

<u>siRNA number</u>	<u>SEQ ID NO</u>	<u>HCV sequence</u>
#12	28	GCTAACACCTCCAGGCCAATACCTGTCTC
#22	29	TCCTTGGTGGCTCCATCTTACCTGTCTC
#32	30	GCTCCATCTTAGCCCTAGTCACCTGTCTC
#42	31	TCTTAGCCCTAGTCACGGCTACCTGTCTC
#52	32	CCTAGTCACGGCTAGCTGTGACCTGTCTC
#62	33	CTAGTCACGGCTAGCTGTGACCTGTCTC
#72	34	CGTGAGCCGCTTGACTGCAGACCTGTCTC
#82	35	GCTGATACTGGCCTCTCTGCACCTGTCTC
#102	36	ACTGGCCTCTCTGCAGATCAACCTGTCTC

20

Several short duplex dsRNAs comprising the HCV sequences identified above in Table 14 (in each case, the first 21 bases constitute conserved HCV sequences of the invention, followed by an 8-base "adapter" sequence, "CCTGTCTC", appended from the Ambion kit used

in synthesis, but which do not appear in the dsRNA effector molecules) targeting the 3'UTR; siRNA #12 targeting the HCV NS5B gene (positive control); the identified HCV core siRNA (positive control); and the identified lamin siRNA (negative control) were synthesized using the Silencer siRNA construction kit, Catalog # 1620 (Ambion Inc., Austin, Tex.). DNA oligonucleotides were synthesized by IDT (Coralville, Iowa).

Table 14A

<u>siRNA number</u>	<u>SEQ ID NO</u>	<u>HCV sequence</u>
#12	<u>63</u>	<u>GCTAAACACTCCAGGCCAATA</u>
#22	<u>64</u>	<u>TCCTTGTTGGCTCCATCTTA</u>
#32	<u>65</u>	<u>GCTCCATCTTAGCCCTAGTCA</u>
#42	<u>66</u>	<u>TCTTAGCCCTAGTCACGGCTA</u>
#52	<u>67</u>	<u>CCTAGTCACGGCTAGCTGTGA</u>
#62	<u>58</u>	<u>CTAGTCACGGCTAGCTGTGAA</u>
#72	<u>69</u>	<u>CGTGAGCCGCTTGACTGCAGA</u>
#82	<u>70</u>	<u>GCTGATACTGGCCTCTCTGCA</u>
#102	<u>71</u>	<u>ACTGGCCTCTCTGCAGATCAA</u>

Several siRNAs comprising the HCV sequences identified above in Table 14 targeting the 3'UTR; siRNA #12 targeting the HCV NS5B gene (positive control); the identified HCV core siRNA (positive control); and the identified lamin siRNA (negative control) were synthesized using the Silencer siRNA construction kit, Catalog # 1620 (Ambion Inc., Austin, Tex.). DNA oligonucleotides were synthesized by IDT (Coralville, Iowa).

Control siRNAs:

1. HCV core (positive control): SEQ ID NO:45
 2. #12, shown in Table 14, targeting the HCV NS5B gene, also a positive control
 3. lamin sequence (negative control): SEQ ID NO:46

Three siRNAs were used as controls: siRNA targeting the cellular gene Lamin for negative control; siRNA targeting the core sequence of HCV as a positive control; siRNA targeting the HCV NS5B gene as a positive control. Two concentrations of each siRNA (9 and 20 pmole) were used and the results were compared with transfection of no siRNA. Accordingly, the Western Blots in FIG. 13 represent 0, 9, and 20 pmoles of the identified siRNAs. siRNA #22, 32, 42, 62, and 72 were notably active in repressing HCV NS5A protein

expression. Presumably, HCV RNA level is also decreased based on the results obtained previously with positive control siRNA for core. Several siRNAs had minimum effect at the concentrations tested and should be evaluated at higher concentrations. These include #12 (targeting NS5B), #102, #52, and #82.

5 Experiment 2

Experiment 2 was performed as described in Experiment 1 of Hepatitis C-Sequences for RNAi Therapeutic Development except that siRNAs R1-R8, comprising the sequences (and their complements) set forth in Table 15 below, were used in transfections. The Western Blot assay performed here was as described in Example 2, Experiment 1. The control HCV core siRNA 10 used as a positive control is the siRNA described in the previous HCV Experiment 1. All siRNAs were transfected at concentrations of 0, 9, and 20 pmole except the control “core” siRNA, which was transfected at levels of 0, 3, and 9 pmole. R1, R2, R3, R5, R7, and R8 all exhibited significant inhibition of HCV as can be seen in the Western Blot, **FIG. 14**.

Table 15

siRNA	SEQ ID NO	HCV sequence
R1	37	CTGGCCTCTCTGCAGATCAAG
R2	38	TGCAGAGAGTGCTGATACTGG
R3	39	TGAGCCGCTTGACTGCAGAGA
R4	40	GAAAGGTCCGTGAGCCGCTT
R5	41	TAGCTGTGAAAGGTCCGTGAG
R6	42	TTAGCCCTAGTCACGGCTAGC
R7	43	TCCATCTTAGCCCTAGTCACG
R8	44	TTGGTGGCTCCATCTTAGCCC

15 All siRNAs evaluated map to the 3'UTR of the HCV genome and are conserved amongst HCV genotypes and quasi-species. SEQ ID NO:27 represents this 101 nt sequence of the HCV 3'UTR, sometimes referred to as the “X” region.

EXAMPLE 3

20 Silencing HBV replication and expression in a replication competent cell culture model

Brief description of cell culture model:

A human liver derived cell line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession #s V01460 and 5 J02203. Following internalization into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirrors HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg (Hepatitis B Surface 10 Antigen) particles into the media of transfected cells. This transfection model reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression and replication.

In this model, cells are co-transfected with the infectious molecular clone of HBV and the individual effector RNA constructs to be evaluated. The cells are then monitored for loss of 15 HBV expression and replication as described below.

The following is an example of an experiment using eiRNA vectors encoding sequences derived from SEQ ID NO:1 and SEQ ID NO:5. The particular eiRNA vectors for this experiment are T7 RNA polymerase-based (See, e.g., the teaching of WO 0063364, with respect to T7 dsRNA expression systems, as well as USSN 60/399,998P, filed 31-Jul-2002 and USSN 20 60/419,532, filed 18-Oct-2002) and encode hairpin RNA structures (especially desirable are, e.g., “forced” hairpin constructs, partial hairpins capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002 and PCT/US2003/024028, filed 31-Jul-2003, as well as the “udderly” structured hairpins (e.g., multi-hairpin long dsRNA vectors and multi-short hairpin structures), hairpins with mismatched 25 regions, and multiepitope constructs as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, filed 20-Oct-2003). It is expected that similar results will be obtained using other expression and promoter systems, e.g., as described above, and/or vectors encoding alternative dsRNA structures (i.e. duplex).

Experimental Procedure: Transfection.

Huh7 cells are seeded into six-well plates such that they are between 80-90% confluence at the time of transfection. All transfections are performed using Lipofectamine™ (Invitrogen) according to the manufacturer's directions. In this experiment, cells are transfected with 50 ng of the infectious HBV plasmid, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid below) 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:1 (described below) and 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:5 (described below). Control cells are transfected with 50 ng of the HBV plasmid and 1 µg of the T7 RNA polymerase expression plasmid. An inert filler DNA, pGL3-basic (Promega, Madison WI), is added to all transfections to bring total DNA/transfection up to 2.5 µg DNA.

Monitoring cells for loss of HBV expression.

Following transfection, cells are monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion and DNA-containing viral particle secretion. Cells are monitored by assaying the media of transfected cells beginning at 2 days post dsRNA administration and every other day thereafter for a period of three weeks. The Auszyme ELISA, commercially available from Abbott Labs (Abbott Park, IL), is used to detect hepatitis B surface antigen (HBsAg). HBsAg is measured since HBsAg is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface antigen cistron in the transfected infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only viral replication but also replication-independent synthesis of HBsAg. Secretion of virion particles containing encapsidated HBV genomic DNA is also measured. Loss of virion particles containing encapsidated DNA is indicative of a loss of HBV replication. Analysis of virion secretion involves a technique that discriminates between naked, immature core particles and enveloped infectious HBV virions [6].

Briefly, pelleted viral particles from the media of cultured cells are subjected to Proteinase K digestion to degrade the core proteins. Following inactivation of Proteinase K, the sample is incubated with RQ1 DNase (Promega, Madison, WI) to degrade the DNA liberated from core particles. The sample is digested again with Proteinase K in the presence of SDS to inactivate the DNase as well as to disrupt and degrade the infectious enveloped virion particle. DNA is

then purified by phenol/chloroform extraction and ethanol precipitated. HBV specific DNA is detected by gel electrophoresis followed by Southern Blot analysis.

Results will desirably indicate a 70-95% decrease in both HBsAg and viral particle secretion in the media of cells transfected with the HBV plasmid, T7 RNA polymerase

- 5 expression plasmid and eiRNA constructs relative to cells transfected with only the HBV plasmid and T7 RNA polymerase expression plasmid.

Vectors used in experiment

Sequence of the T7 RNA polymerase gene

- SEQ ID NO:47 represents the T7 RNA polymerase gene which is cloned into a
10 mammalian expression vector such as pCEP4 (Invitrogen, Carlsbad, CA). Cloning can be easily done by one skilled in the art. One skilled in the art would also be aware that a leader sequence with a Kozak sequence needs to be cloned in directly upstream from the T7 RNA polymerase gene.

eiRNA vector encoding RNA hairpin derived from SEQ ID NO:1

- 15 The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 3004-2950 (about 55 bp) of GenBank accession #s V01460 and J02203. One region of the hairpin encodes the sense version of the sequences and the second region of the hairpin encodes the antisense version of this sequence. Hairpins can easily be designed and made by those skilled in the art.

20 eiRNA vector encoding RNA hairpin derived from SEQ ID NO:5

The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 730-786 of GenBank accession #s V01460 and J02203. The hairpin is designed as described for hairpin encoding sequences from SEQ ID NO:1.

25 EXPERIMENT 1

Rationale for mouse models:

Chimpanzees represent the only animal model in which to study human HBV infectivity. Mouse models are available, however, in which human HBV expression and replication occur. These models have been invaluable for the evaluation of anti-HBV therapeutic agents and have

been shown to be a predictor for the efficacy of these agents in humans [4]. The first of these models are transgenic mouse models, in which the HBV genome or selected HBV genes are expressed [7,8]. Because HBV is integrated into the mouse genome, these animals serve as a model not only for viral replication but also for RT-independent expression of antigen. A similar 5 model exists in which replication competent HBV is expressed transiently from episomal HBV DNA. This model is created by introducing replication competent HBV DNA into mouse liver by hydrodynamic delivery [1]. Unlike the transgenic animals, these mice are not immunotolerant to HBV antigens and immune-mediated clearance of HBV transfected hepatocytes can be studied.

10 Although woodchuck and duck models exist for the study of woodchuck hepatitis (WHBV) and duck hepatitis (DHBV) respectively, we have opted not to use these models for several reasons. 1) Human HBV cannot be studied in these models. As we are ultimately interested in down-regulating expression of human HBV, use of these models would at some point necessitate the re-design and evaluation of vectors and/or RNAs specific for human HBV. 15 2) the mice are isogenic and therefore noise due to genetic variables within the system does not arise. 3) Unlike human HBV, there are no validated WHBV/DHBV cell culture models that can be studied in parallel with their respective animal models.

The experiment described below utilizes hydrodynamic delivery as a method to co-deliver replication competent HBVayw plasmid with the various effector dsRNA (eiRNA) 20 expression vectors. Hydrodynamic delivery is ideal for this experiment because it results in efficient delivery of nucleic acid to the liver [5]. Combination of the dsRNA effector plasmid and replication competent HBV plasmid into the same formulation increases the likelihood that both plasmids are taken up by the same cells. Because expressed effector dsRNA are present in the majority of cells bearing the replicating HBV plasmid, observed results can be attributed to 25 the performance of the effector plasmid rather than to differences in delivery efficiencies. This experiment demonstrates only that a particular eiRNA is efficacious in an infected liver. Formulation and delivery are not addressed by this example. Formulation, dosing and delivery of the eiRNA vector are enabled in the example in which transgenic mice are used.

Experimental procedure:

Control B10.D2 mice are hydrodynamically injected with an infectious molecular clone of HBV (ayw subtype) consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1,2,3]. Following internalization into hepatocytes and nuclear localization, transcription of HBVayw plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication [1]. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the sera of injected animals.

Animals are injected with four doses of the HBV replicon plasmid (1 µg, 3 µg, 5 µg, and 10 µg).

These doses are chosen because they represent non-saturating doses capable of eliciting detectable expression of a reporter plasmid following hydrodynamic delivery. Animals are co-injected with the effector dsRNA expression vector (eiRNA) such that animals in each group receive a 10-19 µg dose of a particular effector construct(s) such that the total DNA dose is 20 µg. For example in mice receiving the 3 µg dose of the HBV replicon, 17 µg of the chosen eiRNA vector(s) is injected for a total of 20 µg injected DNA. The amount of this dose is therefore dependent upon the dose of HBV plasmid used. Control animals are injected with the HBV replicon but not with an eiRNA vector. Control mice are instead co-injected with an inert filler DNA, pGL3-basic (Promega, Madison, WI) such that the total amount of DNA in the formulation is 20 µg. eiRNA vectors in this study are the U6-based expression plasmids, e.g., Ambion, Inc., Austin, TX, USA. These vectors encode short hairpin RNAs derived from SEQ ID NO:1 and SEQ ID NO:4. The exact sequences encoded by these vectors are described below. The vectors are co-injected in equal amounts (by weight). It is expected that similar results will be obtained using other expression and promoter systems as described elsewhere herein and/or vectors encoding alternative structures (i.e. duplex).

Description of U6-based eiRNA vector encoding sequences derived from SEQ ID NO:1: vector encodes a hairpin containing sequences mapping to coordinates 2905-2929 of accession #s V01460 and J02203 (i.e. the hairpin contains the sense and antisense version of this sequence, separated by a loop structure of TTCAAAAGA). Description of U6-based vector sequences can be found in Lee et al. [9]. The second eiRNA vector used in this experiment encodes a hairpin derived from SEQ ID NO:4 and encodes sequences mapping to coordinates 1215-1239 of Accession # V01460 and J02203.

Liver samples are taken from injected animals on day 1 following injection and analyzed for the presence of HBV RNA. This time point has been selected based on published results from Dr. Chisari's laboratory which detail the kinetics of HBVayw plasmid replication in mice following hydrodynamic delivery and demonstrates that peak RNA expression occurs in the liver

5 on day 1 following hydrodynamic delivery [1]. The presence of HBV RNA in liver samples is ascertained by Northern blot analysis. Liver tissue will be evaluated for the down-regulation of HBV RNA expression. In addition, serum will be collected from day 4 mice for measurement of HBVsAg and DNA-containing viral particles. Assays will be as described for the cell culture replicon experiment (Example 3) and as in Yang et al. [1]. Each vector and control group will

10 be comprised of 2 sets of animals, each set corresponding to a collection time point. There are 5 animals in each set.

Results:

Mice that are injected with the HBV replicon and the eiRNA constructs will have

15 decreased HBV-specific RNA, and HBsAg and HBV viral particles as compared to the control animals. In individual animals, decreases will range from about 70% to near 100%.

EXPERIMENT 2

Transgenic mouse studies: Background.

We will be using the HBV transgenic mouse model developed in Dr. Chisari's laboratory [8]. These mice replicate appreciable amounts of HBV DNA and have demonstrated their utility as an antiviral screen that is a predictor of human efficacy [4]. These animals are also ideal in that they are a model for HBV-integrand-mediated expression of antigen and thus can serve as a model not only for viral replication but also for RT-independent expression of antigen. This is important as we are interested in targeting not only viral replication but integrant-mediated antigen expression as well.

These experiments differ from the hydrodynamic delivery experiments in that the effector plasmids are administered to animals using clinically relevant nucleic acid delivery methods. Effectiveness in this model demonstrates efficient delivery of the effector plasmids to mouse

30 hepatocytes.

Experiment.

Mice described in reference [8] will be injected IV with a formulation containing the eiRNA vectors described in the hydrodynamic delivery example. These are the U6-based eiRNA vectors encoding hairpins containing sequences derived from SEQ ID NO:1 and SEQ ID NO:4.

5 Formulation of DNA to be injected.

DNA is formulated with trilactosyl spermine and cholesteryl spermine as described in PCT/US03/14288, "Methods for Delivery of Nucleic Acids", Satishchandran, filed 06-May-2003. Briefly, three formulations are made, all using a charge ratio of 1.2 (positive to negative charge). However, it should noted that formulations with charge ratios between 0.8 and 1.2 are 10 all expected to exhibit efficacy. The DNA starting stock solution for each plasmid is 4 mg/ml. The two plasmid stock solutions are mixed together in equal amounts such that each plasmid is at 2 mg/ml. This plasmid mixture is used for the final formulating. Formulation is as described in PCT/US03/14288 (above): Formulation A) 35% trilactosyl spermine, 65% cholesteryl spermine, Formulation B) 50% trilactosyl spermine, 50% cholesteryl spermine and Formulation C) 80% 15 trilactosyl spermine, 20% cholesteryl spermine. All resultant formulations now contain each plasmid at 1 mg/ml.

Mice are IV injected with 100 µl formulated DNA. One group of mice receives Formulation A, a second group receives Formulation B and a third group receives Formulation C. Three groups of control mice are similarly injected with formulations containing a control 20 DNA, pGL3Basic (Promega, Madison WI), Formulations D, E and F. Injections are carried out once a day for four consecutive days. Injecting for only 1-3 days is efficacious, however, more robust efficacy is seen with a four day injection protocol.

Following administration, HBV RNA and serum levels of HBsAg and DNA containing viral particles will be quantitated on days 5 and 9 post first injection. All analyses will be as 25 described for the hydrodynamic delivery studies.

Results:

HBV-specific RNA levels, HBsAg and virus containing DNA particles will have decreased relative to controls in the Formulation A, B and C groups.

30

EXAMPLE #4

**Silencing HBV replication and expression
in a replication competent cell culture model**

Brief description of cell culture model:

A human liver derived cell line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession AF090840. Following internalization into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the media of transfected cells. This transfection model, reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression and replication.

In this model, cells were co-transfected with the infectious molecular clone of HBV and an eiRNA construct. The cells were then monitored for loss of HBV expression and replication as described below.

The following is an example of an experiment that was performed using an eiRNA vector encoding sequences derived from both SEQ ID NO:1 and SEQ ID NO:2. The particular eiRNA vector used for this experiment is T7 RNA polymerase-based and encodes a duplex RNA of about 650 by (See e.g., WO 00/63364, filed April 19, 2000). It is expected that similar results would be obtained using other expression and promoter systems as described elsewhere herein and/or vectors encoding alternative structures (i.e. duplex).

25

Experimental Procedure: Transfection.

Huh7 cells were seeded into six-well plates such that they were between 80-90% confluence at the time of transfection. All transfections were performed using Lipofectamine™ (InVitrogen) according to the manufacturer's directions. In this experiment, cells were transfected with A) 50 ng of the infectious HBV plasmid adw subtype, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid in Example 3), and 1.5 µg of the HBV-

specific eiRNA vector (described below); B) 50 ng of the infectious HBV plasmid, 1 µg of the T7 RNA polymerase expression plasmid and 1.5 µg of an irrelevant dsRNA expression vector; C) 125 ng of the infectious HBV plasmid, 1 µg of the T7 RNA polymerase expression plasmid and 1.4 µg of the HBV-specific eiRNA vector; and D) 125 ng of the infectious HBV plasmid,

- 5 1 µg of the T7 RNA polymerase expression plasmid and 1.4 µg of an irrelevant dsRNA expression vector. All transfections were carried out in duplicate. In this experiment transfections B and D served as controls. Four days post-transfection, media was removed from transfected cells and assayed for the presence of HBsAg (see below). Media from untransfected cells was also assayed as a background control.

10

Monitoring cells for loss of HBV expression.

Following transfection, cells were monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion. Cells were monitored by assaying the media of transfected cells (and a media control) at 4 days post-dsRNA administration. The Auszyme

- 15 ELISA, commercially available from Abbott Labs (Abbott Park, IL), was used to detect hepatitis B surface antigen (HBsAg). HBsAg was measured since it is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only viral replication but also replication-
- 20 independent synthesis of HB sAg.

Results:

Cells transfected with the HBV-specific eiRNA construct exhibited an 82-93% decrease in HBsAg at the four-day timepoint relative to the control transfections.

25

HBV-specific eiRNA used in this experiment

The eiRNA vector encodes a dsRNA mapping to coordinates 2027–2674 of Gen Bank Accession # AF090840. The sequence therefore includes sequences derived from both SEQ ID NO:1 and SEQ ID NO:2. More specifically, the sequence includes all of SEQ ID NO:2 and 134
30 by derived from SEQ ID NO:1.

EXAMPLE #5The down-regulation of HCV in a cell culture replicon modelBrief description

In this experiment, a cell line is created which expresses functional HCV replicons.

- 5 Creation of the cell line is as detailed in Lohmann et al. [10]. In this experiment Huh7 cells are used as the parental cell line but in theory any human hepatocyte derived cell line can be used. The cells are then transfected with an HCV specific eiRNA vector. The presence of HCV-specific RNA is ascertained by Northern blot analysis as described in Lohmann et al. [10] at days 3-7 post-transfection of eiRNA.

10

Experimental protocol: Transfection.

- Huh7 cells expressing HCV replicons are seeded into six-well plates such that they are between 80-90% confluence at the time of transfection. All transfections are performed using LipofectamineTM (InVitrogen) according to the manufacturer's directions. In this experiment, 15 cells are transfected with 1 µg of a T7 RNA polymerase expression plasmid (plasmid described in Example 3) and 1.5 µg of a T7-based eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:11 (vector described at end of example). Control cells are transfected with 1 µg T7 RNA polymerase expression plasmid and 1.5 µg of the HBV-specific (SEQ ID NO:1 specific) T7-based eiRNA vector described in Example 3. Untransfected 20 replicon-expressing HuH 7 cells are included as a second control. Each transfection mix is made such that ten transfections can be performed/mix resulting in a total of 20 transfections (10 per mix). At days 3, 4, 5, 6, and 7, two wells of cells/each transfection are lysed and RNA is extracted using standard techniques. Samples are analyzed simultaneously by Northern blot analysis for the presence of HCV-specific RNA as described in Lohmann et al. [10].

25

Results

Cells transfected with the HCV-specific eiRNA vector will show decreased HCV-specific RNA levels relative to the control cells at every time-point analyzed.

- 30 HCV-specific eiRNA vector.

The eiRNA vector is T7-based and encodes a hairpin RNA. One side of the hairpin comprises SEQ ID NO:48.

This sequence is followed by a loop structure of 9 Ts. The second side of the hairpin contains a sequence that is complementary to the first side of the hairpin. One skilled in the art
5 can easily design and construct hairpin constructs. Note: it is anticipated that other types of eiRNA vectors driven by other promoters and encoding other types of RNA structures will have similar effects.

EXAMPLE #6

10 Treatment of an HBV/HCV co-infection

Brief description

In this example, cells that are replicating both HBV and HCV replicons are transfected with an eiRNA vector that encodes both HBV and HCV-specific eiRNA.

15 **Experimental protocol:**

Creation of cell lines that contain both HBV and HCV replicons.

HuH 7 cells are first engineered to express functional HCV replicons. Creation of the cell line is as detailed in Lohmann et al. [10]. After cell line establishment, the cells are transfected with an infectious HBV replicon plasmid as described in Example 3 and below in the
20 “Transfection of cells” section. In this example, the replicon is derived from the virus sequence found in Gen Bank Accession #s V01460 and J02203. Theoretically, it is also possible to first create a cell line that stably expresses the HBV replicon and then use this cell line to create one that also expresses HCV replicons. It is also possible to transfect the cells simultaneously with both the HBV and HCV replicons and select and expand cells that are replicating both HBV and
25 HCV replicons.

Transfection of cells.

In this example, the HBV and HCV eiRNAs are encoded by separate cistrons within the same vector. However, similar results are expected if the eiRNAs are encoded within the same
30 cistron or provided by separate vectors. In this example, transcription from each cistron is driven by the T7 RNA polymerase promoter and T7 RNA polymerase. Each promoter is followed by a

hairpin eiRNA which in turn is followed by a T7 terminator (FIG. 1). The cistrons in this example are converging but one could also use diverging cistrons. It should also be noted that one could use other expression systems (including viral) to produce these RNAs and one could also use other promoters, e.g., as described elsewhere herein, to drive expression of these RNAs
5 without significantly affecting efficacy. Selection of the appropriate expression systems and promoters is within the skill in this art. Also one could express other eiRNA structures, e.g., as described elsewhere herein, as well as others, described in the literature in this area. In this example, the HBV eiRNA vector encodes sequences derived from SEQ ID NO:1 and the HCV eiRNA vector encodes sequences derived from SEQ ID NO:11. Description of vector inserts is
10 located at the end of this example.

Huh7 cells are seeded into six-well plates such that they are between 80-90% confluence at the time of transfection. All transfections are performed using LipofectamineTM (Invitrogen) according to the manufacturer's directions. In this experiment, cells are transfected with 50 ng of the infectious HBV plasmid, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid is in Example 3), 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:1 (described below and in Example 3), and 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:11 (described below). Control cells are transfected with 50 ng of the HBV plasmid and 1 µg of the T7 RNA polymerase expression plasmid. An inert filler DNA, pGL3-basic (Promega, Madison WI), is added to all transfections where needed to bring total DNA/transfection up to 2.5 µg DNA. Each transfection mix is made such that ten transfections can be performed/mix resulting in a total of 20 transfections (10 per mix).

Analyses.

25 Following transfection, cells are monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion and DNA-containing viral particle secretion. Cells are monitored by assaying the media of transfected cells beginning at 2 days post dsRNA administration and every other day thereafter for a period of three weeks. The Auszyme ELISA, commercially available from Abbott Labs (Abbott Park, IL), is used to detect hepatitis B surface
30 antigen (HBsAg). HBsAg is measured since it is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected

infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only viral replication but also replication-independent synthesis of HBsAg. Secretion of virion particles containing encapsidated HBV genomic DNA is also measured. Loss of virion particles containing encapsidated DNA is indicative of a loss of
5 HBV replication. Analysis of virion secretion involves a technique that discriminates between naked, immature core particles and enveloped infectious HBV virions [6]. Briefly, pelleted viral particles from the media of cultured cells are subjected to Proteinase K digestion to degrade the core proteins. Following inactivation of Proteinase K, the sample is incubated with RQ1 DNase (Promega, Madison, WI) to degrade the DNA liberated from core particles. The sample is
10 digested again with Proteinase K in the presence of SDS to inactivate the DNase as well as to disrupt and degrade the infectious enveloped virion particle. DNA is then purified by phenol/chloroform extraction and precipitated. HBV specific DNA is detected by gel electrophoresis followed by Southern Blot analysis.

At days 3, 4, 5, 6 and 7, two wells of cells/each transfection (experimental and control)
15 are lysed and RNA is extracted using standard techniques. Samples are also analyzed by Northern blot analysis for the presence of HCV-specific RNA as described in Lohmann et al. [10].

Results.

20 Cells transfected with the HBV-HCV-specific eiRNA vector will show decreased HCV-specific RNA levels relative to the control cells at every time-point analyzed. In addition, the levels of HBsAg and HBV viral particles will also decrease relative to the control transfections.

HCV-specific eiRNA sequence.

25 The eiRNA vector is T7-based and encodes a hairpin RNA. One side of the hairpin comprises SEQ ID NO:48.

~~This sequence is followed by a loop structure of 9 Ts. The second side of the hairpin contains a sequence that is complementary to the first side of the hairpin. One skilled in the art can easily design and construct hairpin constructs. Note: it is anticipated that other types of eiRNA vectors driven by other promoters and encoding other types of RNA structures, including various hairpin structures will have similar effects. Especially desirable are, e.g., "forced"~~

~~hairpin constructs, partial hairpins capable of being extended by RNA dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31 Jul 2002 and PCT/US2003/024028, filed 31 Jul 2003, as well as the “udderly” structured hairpins (e.g., multi-hairpin long dsRNA vectors and multi-short hairpin structures), hairpins with mismatched regions, and multiepitope constructs as taught in USSN 60/419,532, filed 18 Oct 2002, and PCT/US2003/033466, filed 20 Oct 2003, as well as a variety of other dsRNA structures known to those of skill in the art.~~

This sequence is followed by a loop structure of 9 Ts. The second side of the hairpin contains a sequence that is complementary to the first side of the hairpin. One skilled in the art can easily design and construct hairpin constructs. Note: it is anticipated that other types of eiRNA vectors driven by other promoters, including RNA polymerase III promoters, and encoding other types of RNA structures, including various hairpin structures will have similar effects. Especially desirable are, e.g., “forced” hairpin constructs, partial hairpins capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002 and PCT/US2003/024028, filed 31-Jul-2003, as well as the “udderly” structured hairpins (e.g., multi-hairpin long dsRNA vectors and multi-short hairpin structures), hairpins with mismatched regions, and multiepitope constructs as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, filed 20-Oct-2003, as well as a variety of other dsRNA structures known to those of skill in the art.

20

HBV-specific eiRNA-SEQ ID NO:1

The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 3004-2950 (About 55 bp) of GenBank accession #s V01460 and J02203. One region of the hairpin encodes the sense version of the sequences and the second region of the hairpin encodes the antisense version of this sequence. Hairpins can easily be designed and made by those skilled in the art.

EXAMPLE #7

Silencing HBV replication and expression in a replication competent cell culture model (see Example 1) using combinations of HBV- specific eiRNAs in multiple promoter vectors

As disclosed in PCT/US05/29976, filed 23-August-2005 and in U.S. Provisional Applications entitled “Multiple RNA Pol III Promoter Expression Constructs” (Serial No. 60/603622, filed 8/23/04, and Serial No. 60/629942, filed 11/22/04) the teaching of which is hereby incorporated by reference, two or more (preferably 3, 4, 5, 6 or more) of the shRNA

5 sequences shown in Table I and SEQ ID NO:49 may be encoded in the same plasmid vector in separate cistrons under the control of separate promoters for each shRNA. SEQ ID NO:49 is:

GCCTCGCAGACGAAGGTCTCAAGAGAACTTGAGACCTTCGTCTGCGAGGC

10 SEQ ID NO:49 represents the coding strand of a DNA sequence which encodes an shRNA molecule that targets an HBV conserved region. The first 21 bases of the sequence above are identical to the sense sequence of HBV mRNA from position 799 to 779 in the HBV genome, strain AYW (numbered according to the complement strand given in Genbank Accession No. V01460). This sequence is followed by 9 bases (i.e., AGAGAACCTT)representing the loop
 15 portion of the shRNA, followed by 21 bases of the reverse complementary sequence to the first 21 bases. (It will be understood that the loop sequence serves only to join the complementary sequences which form the double-stranded “stem” and therefore considerable variation in length and nucleotide sequence is acceptable within the loop region.) In a preferred embodiment, this DNA sequence will be placed in an appropriate expression vector operably under the control of a
 20 promoter, preferably an RNA polymerase III promoter such 7SK, U6, etc. The resulting RNA transcript:

GCCUCGCAGACGAAGGUCUCAAGAGAACUUUGAGACCUUCGUCUGCGAGGC

25 will assume a hairpin or stem-loop structure having 21 basepairs in a double-stranded conformation.

Using methods commonly employed by one skilled in the art of molecular biology, a single vector encoding two or more, preferably three or more, more preferably four or more, five or more, or all of SEQ ID NO:49, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID
 30 NO:22, and SEQ ID NO:23 is constructed. A particularly preferred embodiment for pharmaceutical applications of dsRNA-mediated silencing of the HBV target comprises a single

expression construct encoding under the control of separate RNA polymerase III promoters, shRNAs corresponding to at least SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:18, and optionally, SEQ ID NO:49 and/or SEQ ID NO:21. Such shRNA-expression vectors may advantageously utilize one or more RNA polymerase III promoters, including U6, 7SK, and H1

5 promoters in several alternative orientations and combinations. Particularly preferred constructs will utilize one or more of the 7SK promoters as taught in U.S. Provisional Serial Nos. 60/603622 and 60/629942. The instant example is thus analogous to Experiment 1 in Example 1 except that instead of introducing one vector with one shRNA at a time, the applicants deliver a single plasmid construct which expresses multiple shRNAs.

10 The advantages of this approach for therapeutic applications of dsRNA silencing are principally in the economy, simplicity and coordinated delivery of a single drug entity which comprises multiple different shRNAs each targeting a different site of the HBV genome. The ability to simultaneously target multiple sites of a viral genome is highly advantageous in preventing the clinically widespread phenomenon of drug resistance (by viral mutation), and the

15 ability to combine dsRNA drug entities against these different target sites in a single delivery agent (the plasmid vectors of this invention) makes this conceptual approach uniquely feasible. While shRNAs, e.g., RNAs corresponding to SEQ ID NO:49, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, and SEQ ID NO:23 may be produced, e.g., through chemical synthesis or *in vitro* expression, and delivered into an animal cell singly and in combination,

20 there are significant advantages in some applications to express within the animal cell multiple shRNAs from a single expression vector. In this example, the potency of the multiple shRNA expression vectors significantly exceeded that of any one of the single vectors used in Experiment 1, as measured by similar assays.

25 **EXAMPLE #8**

Inhibition of Infectious Virions of HCV by dsRNA Effector Molecules

As a further example of HCV-targeted dsRNAs, the sequences given in Table 16 represent highly conserved coding region sequitopes from the 5' and 3' untranslated regions of HCV. Each sequence is written as the coding strand and is used to specify the design of a short hairpin dsRNA effector molecule comprising the coding sequence as shown in Table 16 connected to its reverse complement by a loop or linker sequence as described elsewhere herein.

The sequences shown are predicted to be particularly efficacious as antiviral therapeutic agents because they were tested in a newly available *in vitro* HCV replication system capable of producing whole, infectious virions (disclosed in Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager

5 R, Liang TJ., "Production of infectious hepatitis C virus in tissue culture from a cloned viral genome", Nat Med 2005 Jul;11 (7):791-6; and in Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV., "Robust hepatitis C virus infection in vitro", Proc Natl Acad Sci USA 2005 Jun 28;102(26):9294-9). In this system, all
 10 viral proteins and viral nucleic acid sequences are present in a cell, as in a natural infection. In less complete replicon systems, dsRNA silencing molecules cannot be as rigorously tested as in the new system. It is expected that one or more (2, 3, 4, 5, or more) of the HCV sequitopes and their complements could be utilized as duplex dsRNA effector molecules, short hairpin dsRNA effector molecules, and/or encoded into dsRNA expression vectors capable of expression in vivo in a mammalian cell, including a human cell or organism.

15

Table 16

Seq Name	SEQ ID NO	Sequence (5' to 3')
HCV5M-5.1	72	<u>AAAGGCCTTGTGGTACTGCCT</u>
HCV5M-5.3	73	<u>TTGTGGTACTGCCTGATAGGG</u>
HCVXM-13	74	<u>TAGCTGTGAAAGGTCCGTGAG</u>
HCVXM-34	75	<u>ATCTTAGCCCTAGTCACGGCTAGCTG</u>
HCVXM-35	76	<u>TAGTCACGGCTAGCTGTGAAAGGTCCG</u>

The sequences in Table 17 represent additional preferred highly conserved at least 19 contiguous base pair HCV sequences from the 5' UTR of the virus (SEQ ID NO: 11). To generate the

20 dsRNA effector molecules of the invention, these sequences are used in conjunction with their reverse complement and, optionally, a loop or linker sequence joining the sequence to its reverse complement, when it is desired to form a hairpin dsRNA effector molecule. One or more double-stranded RNA molecules comprising said conserved 5' UTR sequences (from SEQ ID NO: 11) may advantageously be used in combination with one or more other dsRNA effector
 25 molecules of the invention, including e.g., one or more highly conserved sequences from the 3' UTR (SEQ ID NO:27) and/or one or more at least 19 contiguous base pair sequences from SEQ ID NO. 12.

Table 17

<u>HCV 5' UTR siRNAs</u>		
<u>Sequence Name</u>	<u>Sequence (5' to 3')</u>	<u>SEQ ID NO</u>
<u>HCV5P-1.1</u>	<u>CCTGTGAGGAACACTACTGTCTT</u>	<u>77</u>
<u>HCV5P-1.2</u>	<u>ACGCAGAAAGCGTAGCCAT</u>	<u>78</u>
<u>HCV5P-1.3</u>	<u>CGTCTAGCCATGGCGTTAGTA</u>	<u>79</u>
<u>HCV5P-1.4</u>	<u>GTCTAGCCATGGCGTTAGTAT</u>	<u>80</u>
<u>HCV5P-1.5</u>	<u>CTCCCCCTGTGAGGAACACTACTGTCTT</u>	<u>81</u>
<u>HCV5P-1.6</u>	<u>GAGGAACACTACTGTCTTCACGCAGAA</u>	<u>82</u>
<u>HCV5P-1.7</u>	<u>GTGAGGAACACTACTGTCTTCACGCAGAA</u>	<u>83</u>
<u>HCV5P-2.1</u>	<u>GAGCCATAGTGGTCTGCCGAA</u>	<u>84</u>
<u>HCV5P-2.2</u>	<u>GAACCGGTGAGTACACCGGAA</u>	<u>85</u>
<u>HCV5P-2.3</u>	<u>ACCGGTGAGTACACCGGAAAT</u>	<u>86</u>
<u>HCV5P-2.4</u>	<u>GGGAGAGCCATAGTGGTCTGCCGAA</u>	<u>87</u>
<u>HCV5P-5.1</u>	<u>GGCCTTGTGGTACTGCCGTGAT</u>	<u>88</u>
<u>HCV5P-5.2</u>	<u>GCCTTGTGGTACTGCCGTGATA</u>	<u>89</u>
<u>HCV5P-5.3</u>	<u>GTACTGCCGTGATAGGGTGCTT</u>	<u>90</u>
<u>HCV5P-5.4</u>	<u>AAGGCCTTGTGGTACTGCCGTGATAGGG</u>	<u>91</u>
<u>HCV5P-5.5</u>	<u>CGAAAGGCCTTGTGGTACTGCCGTGATA</u>	<u>92</u>
<u>HCV5P-5.6</u>	<u>CTTGCAGTGCCCCGGGAGGTCTCGTA</u>	<u>93</u>
<u>HCV5M-1.1</u>	<u>ATCACTCCCCTGTGAGGAAC</u>	<u>94</u>
<u>HCV5M-1.2</u>	<u>TTCACGCAGAAAGCGTCTAGC</u>	<u>95</u>
<u>HCV5M-1.3</u>	<u>TAGCCATGGCGTTAGTATGAG</u>	<u>96</u>
<u>HCV5M-1.4</u>	<u>ATCACTCCCCTGTGAGGAACACTG</u>	<u>97</u>
<u>HCV5M-1.5</u>	<u>ATCACTCCCCTGTGAGGAACACTGTG</u>	<u>98</u>
<u>HCV5M-1.6</u>	<u>AACTACTGTCTTCACGCAGAAAGCG</u>	<u>99</u>
<u>HCV5M-1.7</u>	<u>AACTACTGTCTTCACGCAGAAAGCGTC</u>	<u>100</u>
<u>HCV5M-2.1</u>	<u>ATAGTGGTCTGCCGAACCGGT</u>	<u>101</u>
<u>HCV5M-2.2</u>	<u>TAGTGGTCTGCCGAACCGGT</u>	<u>102</u>
<u>HCV5M-2.3</u>	<u>AACCGGTGAGTACACCGGAATTGCC</u>	<u>103</u>
<u>HCV5M-5.2</u>	<u>AAGGCCTTGTGGTACTGCCGT</u>	<u>104</u>
<u>HCV5M-5.4</u>	<u>TACTGCCGTGATAGGGTGCTTG</u>	<u>105</u>
<u>HCV5M-5.5</u>	<u>TTGTGGTACTGCCGTGATAGGGTGCTTG</u>	<u>106</u>
<u>HCV5M-5.6</u>	<u>TACTGCCGTGATAGGGTGCTTGCGAG</u>	<u>107</u>
<u>HCV5M-5.7</u>	<u>TAGGGTGCTTGCAGTGCCCCGGG</u>	<u>108</u>
<u>HCV5M-5.8</u>	<u>TTGCGAGTGCCCCGGGAGGTCTCGTAG</u>	<u>109</u>

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TITLE
CONSERVED HBV AND HCV SEQUENCES USEFUL
FOR GENE SILENCING

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application is a Continuation-in-part under 35 USC §120 of International Application PCT/US2004/019229, filed June 10, 1994, which claims the benefit of priority of U.S. Provisional Application 60/478,076, filed June 12, 2003, the entireties of both of which are incorporated herein by reference in their entireties. This application also claims the benefit under 35 U.S.C. §119(e) of U.S. Provisional Application No. 60/638,294, filed December 22,
10 2004, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

This invention relates to methods and compositions utilizing conserved genetic sequences of known hepatitis B viral (HBV) strains and known hepatitis C viral (HCV) strains to modulate the expression of HBV and/or HCV in mammalian cells, via double-stranded RNA-mediated
15 gene silencing, including post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS).

BACKGROUND OF THE INVENTION

Human hepatitis C (HCV) is a major public health problem with an estimated 200 million persons worldwide infected. The number of new infections per year in the United States is
20 estimated to be about 25,000 in 2001. This number has declined from an estimated 240,000 new cases per year in the 1980's due to blood donor screening. Nevertheless, an estimated 3.9 million (1.8%) Americans have been infected with HCV, of whom 2.7 million are chronically infected.. Hepatitis C shows significant genetic variation in worldwide populations, evidence of its frequent rates of mutation and rapid evolution. There are six basic genotypes of HCV, with
25 15 recorded subtypes, which vary in prevalence across different regions of the world. Each of these major genotypes may differ significantly in their biological effects - in terms of replication, mutation rates, type and severity of liver damage, and detection and treatment options - however, these differences are not yet clearly understood.

There is currently no vaccine against HCV and available drug therapy, including ribavirin
30 and interferon, is only partially effective. It is estimated that 75-85% of infected persons will

develop a chronic infection, with 70% of chronically infected persons expected to develop chronic liver disease including hepatocellular carcinoma. Chronic HCV related liver disease is a leading indication for liver transplant.

Although a human hepatitis B vaccine has been available since 1982, it is estimated that 5 350 million persons worldwide are chronically infected with HBV. While the number of new infections per year in the United States has declined from an average of 260,000 in the 1980s to about 78,000 in 2001, there are an estimated 1.25 million hepatitis B carriers, defined as persons positive for hepatitis B surface antigen (HBsAg) for more than 6 months. Such carriers of HBV 10 are at increased risk for developing cirrhosis, hepatic decompensation, and hepatocellular carcinoma. Although most carriers do not develop hepatic complications from chronic hepatitis B, 15% to 40% will develop serious sequelae during their lifetime, and death from chronic liver disease occurs in 15-25% of chronically infected persons.

There is a need for improved therapeutic agents effective in patients suffering from HBV and/or HCV infection, especially chronic infection, which together are estimated to account for 15 75% of all cases of liver disease around the world. There is also an extreme need for prophylactic methods and agents effective against HCV.

Nucleic acids (e.g., DNA, RNA, hybrid, heteroduplex, and modified nucleic acids) have come to be recognized as extremely valuable agents with significant and varied biological activities, including their use as therapeutic moieties in the prevention and/or treatment of 20 disease states in man and animals. For example, oligonucleotides acting through antisense mechanisms are designed to hybridize to target mRNAs, thereby modulating the activity of the mRNA. Another approach to the utilization of nucleic acids as therapeutics is designed to take advantage of triplex or triple strand formation, in which a single-stranded oligomer (e.g., DNA or RNA) is designed to bind to a double-stranded DNA target to produce a desired result, e.g., 25 inhibition of transcription from the DNA target. Yet another approach to the utilization of nucleic acids as therapeutics is designed to take advantage of ribozymes, in which a structured RNA or a modified oligomer is designed to bind to an RNA or a double-stranded DNA target to produce a desired result, e.g., targeted cleavage of RNA or the DNA target and thus inhibiting its expression. Nucleic acids may also be used as immunizing agents, e.g., by introducing DNA 30 molecules into the tissues or cells of an organism that express proteins capable of eliciting an immune response. Nucleic acids may also be engineered to encode an RNA with antisense,

ribozyme, or triplex activities, or to produce RNA that is translated to produce protein(s) that have biological function.

More recently, the phenomenon of RNAi or double-stranded RNA (dsRNA)-mediated gene silencing has been recognized, whereby dsRNA complementary to a region of a target gene
5 in a cell or organism inhibits expression of the target gene (see, e.g., WO 99/32619, published 1 July 1999, Fire et al.; and U.S. 6,506,559: "Genetic Inhibition by Double-Stranded RNA;" WO 00/63364: "Methods and Compositions for Inhibiting the Function of Polynucleotide Sequences," Pachuk and Satischchandran; and U.S.S.N. 60/419,532, filed October 18, 2002).
10 dsRNA-mediated gene silencing, utilizing compositions providing an at least partially double-stranded dsRNA, is expected to provide extremely valuable therapeutic and/or prophylactic agents against viral infection, including HBV and/or HCV, including in the extremely difficult problem of chronic HBV and/or HCV infection.

SUMMARY OF THE INVENTION

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in
15 an *in vivo* mammalian cell comprising administering to said cell at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6, or more double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T. In a preferred method, three or
20 four dsRNA effector molecules, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T; are administered to an *in vivo* mammalian cell. The double-stranded RNA effector molecules may be prepared exogenously and administered into a mammalian cell or expressed intracellularly in a mammalian cell from a double-stranded RNA expression vector,
25 i.e., an expression vector engineered to express a dsRNA effector molecule in a mammalian cell. In a preferred method, at least three or four dsRNA effector molecules, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T; are encoded in a dsRNA expression vector which is administered to an *in vivo* mammalian cell.

A composition for inhibiting the expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising at least one, preferably 2, 3, 4, 5, 6 or more double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, 5 SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T. In a preferred composition, at least three or four dsRNA effector molecules are included, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T. The double-stranded RNA effector molecules may be prepared exogenenously and the composition 10 comprising two, three, four, five, six, or more dsRNA effector molecules administered into a mammalian cell, or the composition may comprise one or more dsRNA expression constructs capable of expressing in a mammalian cell two, three, four, five, six or more of said dsRNA effector molecules. In a preferred composition, three or four dsRNA effector molecules, each comprising a sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19, 15 SEQ ID NO:23, and SEQ ID NO:49; wherein U is substituted for T, are encoded in a dsRNA expression vector.

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell at least two, preferably 3, 4, 5, 6 or more, double-stranded RNA effector molecules, each double-stranded RNA effector molecule 20 comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In a preferred method, said dsRNA effector molecules will comprise 3 or 4 sequences selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 25 55, SEQ ID NO:59, and SEQ ID NO:62; wherein U is substituted for T. The double-stranded RNA effector molecules may be stem-loop or hairpin structures and/or duplex double-stranded RNA molecules. The double-stranded RNA effector molecules may be prepared 30 exogenenously and the two, three, four, five, six, or more dsRNA effector molecules administered into a mammalian cell, or one or more dsRNA expression constructs capable of expressing in a mammalian cell two, three, four, five, six or more of said dsRNA effector molecules may be administered.

A composition for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising at least two double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In a preferred composition, three or four of said dsRNA effector molecules will be included, or encoded in an expression vector, comprising sequences selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:59, and SEQ ID NO:62; wherein U is substituted for T. The double-stranded RNA effector molecules may be prepared exogenously and the composition will comprise two, three, four, five, six, or more of said dsRNA effector molecules for administration into a *in vivo* mammalian cell, or the composition may comprise one or more dsRNA expression constructs capable of expressing in a mammalian cell two, three, four, five, six or more of said dsRNA effector molecules.

15 In another aspect the invention relates to methods and compositions for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell at least two, preferably 3, 4, 5, 6 or more, double-stranded RNA effector molecules, each double-stranded RNA effector molecule comprising: (a) a sequence selected from the group consisting of SEQ ID NO: 50; SEQ ID NO: 51; SEQ ID NO: 52; SEQ ID NO:53; SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56; SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO: 60; SEQ ID NO:61; and SEQ ID NO:62; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T.

A polynucleotide sequence comprising SEQ ID NO:49.

25 A method for inhibiting expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering to said cell at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6, or more double-stranded RNA effector molecules, comprising (a) an RNA sequence equivalent to a hepatitis C virus DNA coding strand sequence selected from the group consisting of sequence position 9510-9531, 9510-9533, 9510-9534, 9510-9535, 9510-9536, 9514-9534, 9514-9535, 9514-9536, 9514-9539, 9514-9540, 9514-9542, 9517-9539, 9517-9540, 9517-9542, 9517-9544, 9518-9539, 9518-9540, 9518-9542, 9518-

9544, 9520-9540, 9520-9542, 9520-9544, 9520-9548, 9521-9542, 9521-9544, 9521-9548, 9521-
9549, 9522-9542, 9522-9544, 9522-9548, 9522-9549, 9527-9548, 9527-9549, 9527-9551, 9527-
9552, 9527-9553, 9527-9555, 9528-9548, 9528-9549, 9528-9551, 9528-9552, 9528-9553, 9528-
9555, 9530-9551, 9530-9552, 9530-9553, 9530-9555, 9530-9557, 9530-9558, 9532-9552, 9532-
5 9553, 9532-9555, 9532-9557, 9532-9558, 9532-9559, 9532-9560, 9537-9557, 9537-9558, 9537-
9559, 9537-9560, 9537-9561, 9537-9564, 9538-9558, 9538-9559, 9538-9560, 9538-9561, 9538-
9564, 9538-9566, 9541-9561, 9541-9564, 9541-9566, 9541-9568, 9541-9569, 9543-9564, 9543-
9566, 9543-9568, 9543-9569, 9543-9571, 9545-9566, 9545-9568, 9545-9569, 9545-9571, 9545-
10 9573, 9546-9564, 9546-9566, 9546-9569, 9546-9571, 9546-9573, 9547-9568, 9547-9569, 9547-
9571, 9547-9573, 9547-9575, 9550-9571, 9550-9573, 9550-9575, 9550-9577, 9550-9578, 9554-
9575, 9554-9577, 9554-9578, 9554-9580, 9556-9577, 9556-9578, 9556-9580, 9556-9584, 9562-
9584, 9562-9586, 9562-9587, 9562-9588, 9562-9589, 9563-9584, 9563-9586, 9563-9587, 9563-
9588, 9563-9589, 9563-9591, 9565-9586, 9565-9587, 9565-9588, 9565-9589, 9565-9591, 9565-
15 9593, 9567-9587, 9567-9588, 9567-9589, 9567-9591, 9567-9593, 9567-9595, 9570-9591, 9570-
9593, 9570-9595, 9570-9596, 9570-9598, 9572-9593, 9572-9595, 9572-9596, 9572-9598, 9574-
9595, 9574-9596, 9574-9598, 9574-9601, 9576-9596, 9576-9598, 9576-9601, 9576-9604, 9579-
9601, 9579-9604, 9581-9601, 9581-9604, and 9583-9604 and (b) an RNA sequence which is the
reverse complement of the selected sequence equivalent to the hepatitis C virus DNA coding
strand sequence. In some embodiments, said RNA sequences (a) and (b) are linked by a loop
20 sequence and the double-stranded RNA effector molecule(s) forms a stem-loop or hairpin
dsRNA structure. In some aspects, said double-stranded RNA effector molecule(s) are duplex
dsRNAs, formed from two separate RNA strands. In some aspects, the method involves
administering to a mammalian cell an expression construct encoding one, two, three, four, five or
more of said dsRNA effector molecules. In some embodiments designed to target the HCV
25 minus strand, the dsRNA effector molecule will comprise (a) an RNA sequence corresponding to
a hepatitis C virus DNA coding strand sequence as specified above, and (b) the reverse
complement of said RNA sequence, optionally linked by a loop sequence. In some
embodiments, the dsRNA effector molecule(s) is encoded by an expression construct.

In some aspects the invention relates to a composition for inhibiting the expression of a
30 polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising at least
one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6 or more double-stranded

RNA effector molecules, or a dsRNA expression construct capable of transcribing one, 2, 3, 4, 5, 6 or more of said dsRNA effector molecules in an in vivo mammalian cell, each of said dsRNA effector molecules comprising (a) an RNA sequence equivalent to a hepatitis C virus DNA coding strand sequence selected from the group consisting of sequence position 9510-9531,

5 9510-9533, 9510-9534, 9510-9535, 9510-9536, 9514-9534, 9514-9535, 9514-9536, 9514-9539, 9514-9540, 9514-9542, 9517-9539, 9517-9540, 9517-9542, 9517-9544, 9518-9539, 9518-9540, 9518-9542, 9518-9544, 9520-9540, 9520-9542, 9520-9544, 9520-9548, 9521-9542, 9521-9544, 9521-9548, 9521-9549, 9522-9542, 9522-9544, 9522-9548, 9522-9549, 9527-9548, 9527-9549, 9527-9551, 9527-9552, 9527-9553, 9527-9555, 9528-9548, 9528-9549, 9528-9551, 9528-9552,

10 9528-9553, 9528-9555, 9530-9551, 9530-9552, 9530-9553, 9530-9555, 9530-9557, 9530-9558, 9532-9552, 9532-9553, 9532-9555, 9532-9557, 9532-9558, 9532-9559, 9532-9560, 9537-9557, 9537-9558, 9537-9559, 9537-9560, 9537-9561, 9537-9564, 9538-9558, 9538-9559, 9538-9560, 9538-9561, 9538-9564, 9538-9566, 9541-9561, 9541-9564, 9541-9566, 9541-9568, 9541-9569, 9543-9564, 9543-9566, 9543-9568, 9543-9569, 9543-9571, 9545-9566, 9545-9568, 9545-9569,

15 9545-9571, 9545-9573, 9546-9564, 9546-9566, 9546-9569, 9546-9571, 9546-9573, 9547-9568, 9547-9569, 9547-9571, 9547-9573, 9547-9575, 9550-9571, 9550-9573, 9550-9575, 9550-9577, 9550-9578, 9554-9575, 9554-9577, 9554-9578, 9554-9580, 9556-9577, 9556-9578, 9556-9580, 9556-9584, 9562-9584, 9562-9586, 9562-9587, 9562-9588, 9562-9589, 9563-9584, 9563-9586, 9563-9587, 9563-9588, 9563-9589, 9563-9591, 9565-9586, 9565-9587, 9565-9588, 9565-9589,

20 9565-9591, 9565-9593, 9567-9587, 9567-9588, 9567-9589, 9567-9591, 9567-9593, 9567-9595, 9570-9591, 9570-9593, 9570-9595, 9570-9596, 9570-9598, 9572-9593, 9572-9595, 9572-9596, 9572-9598, 9574-9595, 9574-9596, 9574-9598, 9574-9601, 9576-9596, 9576-9598, 9576-9601, 9576-9604, 9579-9601, 9579-9604, 9581-9601, 9581-9604, and 9583-9604 and (b) the reverse complement of said selected RNA sequence equivalent to the hepatitis C virus DNA coding strand sequence. In some embodiments, said RNA sequences (a) and (b) are linked by a loop sequence, and the double-stranded RNA effector molecule(s) is a single RNA strand which forms a stem-loop or hairpin dsRNA structure. In other embodiments, the dsRNA effector molecule(s) is a duplex dsRNA molecule formed from two separate strands of RNA.

25

In another aspect, the invention relates to compositions for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an in vivo mammalian cell comprising at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6 or more double-stranded

RNA effector molecules, or a dsRNA expression construct capable of expressing one, 2, 3, 4, 5, 6 or more of said dsRNA effector molecules in an in vivo mammalian cell, each of said dsRNA effector molecules comprising (a) a sequence selected from the group consisting of SEQ ID NO: 63; SEQ ID NO: 64; SEQ ID NO: 65; SEQ ID NO:66; SEQ ID NO: 67, SEQ ID NO: 68, SEQ
5 ID NO: 69; SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO: 73; SEQ ID NO:74; SEQ ID NO:75; and SEQ ID NO:76; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In certain preferred embodiments, the sequence is selected from the group consisting of SEQ ID NO:72; SEQ ID NO: 73; SEQ ID NO:74; SEQ ID NO: 75; and SEQ ID NO:76.

10 In another aspect, the invention relates to methods for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an in vivo mammalian cell comprising administering at least one double-stranded RNA effector molecule, preferably 2, 3, 4, 5, 6 or more double-stranded RNA effector molecules, or a dsRNA expression construct capable of expressing one, 2, 3, 4, 5, 6 or more of said dsRNA effector molecules in an in vivo mammalian
15 cell, each of said dsRNA effector molecules comprising (a) a sequence selected from the group consisting of SEQ ID NO: 63; SEQ ID NO: 64; SEQ ID NO: 65; SEQ ID NO:66; SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69; SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO: 73; SEQ ID NO:74; SEQ ID NO:75; and SEQ ID NO:76; (b) the reverse complement of said selected sequence; and (c) optionally, a sequence linking sequences (a) and (b); wherein U is substituted for T. In certain preferred embodiments, the sequence is selected from the group consisting of SEQ ID NO:72; SEQ ID NO: 73; SEQ ID NO:74; SEQ ID NO: 75; and SEQ ID NO:76.
20

25 In another aspect, the invention relates to a polynucleotide sequence comprising an RNA sequence equivalent to and/or complementary to a hepatitis C virus DNA coding strand sequence selected from the group consisting of sequence position 9510-9531, 9510-9533, 9510-9534, 9510-9535, 9510-9536, 9514-9534, 9514-9535, 9514-9536, 9514-9539, 9514-9540, 9514-9542, 9517-9539, 9517-9540, 9517-9542, 9517-9544, 9518-9539, 9518-9540, 9518-9542, 9518-9544, 9520-9540, 9520-9542, 9520-9544, 9520-9548, 9521-9542, 9521-9544, 9521-9548, 9521-9549, 9522-9542, 9522-9544, 9522-9548, 9522-9549, 9527-9548, 9527-9549, 9527-9551, 9527-9552,
30 9527-9553, 9527-9555, 9528-9548, 9528-9549, 9528-9551, 9528-9552, 9528-9553, 9528-9555, 9530-9551, 9530-9552, 9530-9553, 9530-9555, 9530-9557, 9530-9558, 9532-9552, 9532-9553,

9532-9555, 9532-9557, 9532-9558, 9532-9559, 9532-9560, 9537-9557, 9537-9558, 9537-9559,
9537-9560, 9537-9561, 9537-9564, 9538-9558, 9538-9559, 9538-9560, 9538-9561, 9538-9564,
9538-9566, 9541-9561, 9541-9564, 9541-9566, 9541-9568, 9541-9569, 9543-9564, 9543-9566,
9543-9568, 9543-9569, 9543-9571, 9545-9566, 9545-9568, 9545-9569, 9545-9571, 9545-9573,
5 9546-9564, 9546-9566, 9546-9569, 9546-9571, 9546-9573, 9547-9568, 9547-9569, 9547-9571,
9547-9573, 9547-9575, 9550-9571, 9550-9573, 9550-9575, 9550-9577, 9550-9578, 9554-9575,
9554-9577, 9554-9578, 9554-9580, 9556-9577, 9556-9578, 9556-9580, 9556-9584, 9562-9584,
9562-9586, 9562-9587, 9562-9588, 9562-9589, 9563-9584, 9563-9586, 9563-9587, 9563-9588,
9563-9589, 9563-9591, 9565-9586, 9565-9587, 9565-9588, 9565-9589, 9565-9591, 9565-9593,
10 9567-9587, 9567-9588, 9567-9589, 9567-9591, 9567-9593, 9567-9595, 9570-9591, 9570-9593,
9570-9595, 9570-9596, 9570-9598, 9572-9593, 9572-9595, 9572-9596, 9572-9598, 9574-9595,
9574-9596, 9574-9598, 9574-9601, 9576-9596, 9576-9598, 9576-9601, 9576-9604, 9579-9601,
9579-9604, 9581-9601, 9581-9604, and 9583-9604.

Applicants' invention further provides a method for inhibiting expression of a
15 polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising
administering to said cell a double-stranded RNA effector molecule comprising an at least 19
contiguous base pair nucleotide sequence from within a sequence selected from the group
consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ
ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is
20 substituted for T. In a preferred embodiment of the method, effector sequences from more than
one SEQ ID sequence may be administered to the same cell, and/or more than one effector
sequence from within the same SEQ ID sequence may be administered to the same cell.

Applicants further provide a method for inhibiting expression of a polynucleotide
sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering to said cell
25 a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair
nucleotide sequence from within a sequence selected from the group consisting of SEQ ID
NO:11, SEQ ID NO:12, and SEQ ID NO:27; wherein U is substituted for T. In a preferred
embodiment of this aspect of the method, effector molecules from both SEQ ID NO:11 and SEQ
ID NO:12 may be administered to the same cell; or from both SEQ ID NO: 11 and SEQ ID
30 NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from each of SEQ ID NO: 11, SEQ

ID NO:12, and SEQ ID NO:27, are administered to the same cell; and/or more than one effector molecule from within the same SEQ ID NO may be administered to the same cell.

Applicants further provide a method for inhibiting expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in the same *in vivo* mammalian cell, comprising administering to said cell a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:27; wherein U is substituted for T. In preferred embodiments of this aspect of the invention, effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 may be administered to the same cell; and/or effector molecules from both SEQ ID NO:11 and SEQ ID NO:12; or from both SEQ ID NO: 11 and SEQ ID NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from SEQ ID NO: 11, SEQ ID NO:12 and SEQ ID NO:27; may be administered to the same cell; and/or more than one effector molecules from within the same SEQ ID NO may be administered to the same cell.

Applicants further provide a composition for inhibiting the expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO is present in the composition.

Applicants further provide a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ

ID NO:12 and SEQ ID NO:27; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 are present in the composition; or from both SEQ ID NO: 11 and SEQ ID NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from each of SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO:27, are present in the same composition, and/or wherein more than one effector molecule from within the same SEQ ID NO may be present in the composition.

- Applicants further provide a composition for inhibiting the expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in a single *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12 and SEQ ID NO:27; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12; or from both SEQ ID NO: 11 and SEQ ID NO:27; or from both SEQ ID NO: 12 and SEQ ID NO:27; or from each of SEQ ID NO: 11, SEQ ID NO:12, and SEQ ID NO:27, are present in the composition; and/or wherein more than one effector sequence from within the same SEQ ID NO may be present in the composition.

In particularly preferred embodiments of the above methods and compositions of the invention, the polynucleotide sequence is present within a double-stranded region of an RNA, and the mammalian cell is a human cell.

Further provided are compositions for inhibiting the expression of a polynucleotide sequence of hepatitis B virus and/or a polynucleotide sequence of hepatitis C virus in mammalian cells, wherein said compositions comprise an at least 19 contiguous nucleotide sequence selected from within SEQ ID NO:1 through SEQ ID NO:12, and SEQ ID NO:27; the complement sequences of said SEQ ID NO:1 through SEQ ID NO:12, and SEQ ID NO: 27 sequences, and mixtures of these sequences. In this embodiment of the invention, the “an at least

19 contiguous nucleotide sequence" is preferably DNA, and the mammalian cell is preferably human. Also provided are expression constructs comprising any of the aforementioned compositions and a mammalian cell comprising said expression constructs.

Another aspect provides for a polynucleotide sequence comprising a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for polynucleotide sequence comprising nucleotides 1-19, 1-20, 1-21, 2-20, 2-21, or 3-21 of a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for a polynucleotide sequence comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from SEQ ID NO:27 through SEQ ID NO:44.

Another aspect provides a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in a mammalian cell, comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:27; wherein U is substituted for T.

In various aspects of the foregoing methods and compositions, the in vivo mammalian cell is an in vivo human cell.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 through SEQ ID NO:10 represent conserved regions of the hepatitis B genome.

SEQ ID NO:11 and SEQ ID NO:12 represent conserved regions of the hepatitis C genome.

SEQ ID NO:13 represents the nucleotide sequence of human U6 promoter.

SEQ ID NO:14 and SEQ ID NO:15 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:5.

SEQ ID NO:16 and SEQ ID NO:17 represent eiRNAs that have HBV 20 sequences mapping within SEQ ID NO:4.

SEQ ID NO:18 represents eiRNA that has an HBV sequence mapping within SEQ ID NO:10.

SEQ ID NO:19 through SEQ ID NO:22 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:3.

SEQ ID NO:23 and SEQ. ID NO:24 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:2.

SEQ ID NO:25 and SEQ ID NO:26 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:1.

SEQ ID NO:27 represents the “X” region of the HCV 3’UTR.

SEQ ID NO:28 through SEQ ID NO:36 represent siRNAs mapping to the the HCV 3’UTR.

5 SEQ ID NO:37 through SEQ ID NO:44 represent siRNAs mapping to the “X” region of the HCV 3’UTR.

SEQ ID NO:45 represents an siRNA mapping to the HCV core.

SEQ ID NO:46 represents an siRNA mapping to lamin.

SEQ ID NO:47 represents the T7 RNA polymerase gene.

10 SEQ ID NO:48 represents a 5’ segment of the hepatitis C virus sequence (corresponds to positions 36 to 358 in Genbank Accession Number AJ238799, with 2 base changes, C to G at AJ238799 position 204 and G to A at AJ238799 position 357).

SEQ ID NO:49 represents an eiRNA (shRNA) molecule to a conserved HBV sequence.

SEQ ID NO:50 through SEQ ID NO:62 represent the first 21 nucleotides of SEQ ID NOs: 14-23,

15 25-26, and 49.

SEQ ID NO:63 through SEQ ID NO:71 represent the first 21 nucleotides of SEQ ID NOs: 28-36.

SEQ ID NO:72 through SEQ ID NO:76 represent highly conserved coding region sequitopes from the 5’ and 3’ untranslated regions of HCV.

SEQ ID NO:77 through SEQ ID NO:109 represent highly conserved HCV sequences from the 5’

20 UTR of the HCV (SEQ ID NO: 11).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a vector illustrating placement of the T7 RNA polymerase promoter and T7 RNA polymerase, showing inclusion of hairpin eiRNA sequences.

25 Figure 2 is a graph showing HBsAg inhibition corresponding to data 15 found in Table 2.

Figure 3 is a graph showing HBsAg inhibition corresponding to data found in Table 3.

Figure 4 is a graph showing HBsAg inhibition corresponding to data found in Table 4.

Figure 5 is a graph showing HBsAg inhibition corresponding to data found in Table 5.

Figure 6 is a graph showing HBsAg inhibition corresponding to data found in Table 6.

Figure 7 is a graph showing HBsAg inhibition corresponding to data found in Table 7.

Figure 8 is a graph showing HBsAg inhibition corresponding to data found in Table 8.

5 Figure 9 is a drawing depicting effective HBV-AYW shRNA inserts.

Figure 10 is a graph showing HBsAg inhibition corresponding to data found in Table 9.

Figure 11 is a bar graph showing downregulation of HBV RNA by Northern Blot analysis.

10 Figure 12 is a graph showing HBsAg inhibition corresponding to data found in Table 12.

Figure 13 is a Western Blot showing levels of HCV NS5A protein at (l to r) 0, 9, and 20 pmole of the identified siRNAs, as described in more detail in Experiment 1 of Example 2.

15 Figure 14 is a Western Blot showing levels of HCV NS5A protein at 20 (I to r) 0, 9, and 20 pmole of the identified siRNA, and 0, 3, and 9 pmole of the “core” positive control siRNA, as described in more detail in Experiment 2 of Example 2.

20 Figure 15 is a table of additional conserved HCV genome sequence segments suitable for generating dsRNA effector molecules which inhibit the expression of polynucleotide sequences of hepatitis C virus, including expressed shRNA for gene silencing. Each sequence represents a DNA coding strand sequence in standard 5' to 3' polarity which (together with its reverse complement) can be utilized to transcribe or design a double-stranded RNA effector molecule, e.g., an shRNA or duplex dsRNA molecule targeted to degrade the negative strand of HCV RNA. E.g., an DNA sequence, followed by a loop sequence (e.g., a 9 base loop sequence as described elsewhere herein), followed by the reverse complement of the sequence given in the table, may be incorporated into an expression construct under the control of an appropriate promoter. The shRNA molecule transcribed from such an expression construct is expected to

inhibit expression of HCV polynucleotide sequences and/or mediate dsRNA silencing of HCV. For example, in the case of the 22 base sequence shown for positions 9545-9566, a construct is made to contain a 53 bp insert, comprising the 22 base sequence of 9545-9566, a linker or loop sequence, and the reverse complement of the 9545-9566 sequence, preferably under the control
5 of an RNA polymerase III promoter and ending with an RNA polymerase III terminator, e.g., a run of 4, 5, or more T residues. The RNA equivalent of this sequence, having U's instead of T's, would read (in the 5' to 3' direction):

AAAGGUCCGUGAGCCGCUUGAC-XXXXXX-
GUCAAGCGGUCACGGACCUU U

10 where X represents bases of the loop that are unable to form stable base pairs with any other portion of the 53 bp shRNA sequence. The loop may vary considerably, however, as to both length and nucleotide sequence, so long as the formation of the double-stranded "stem" region of the hairpin is not adversely affected. Thus, in expression constructs that are the subject of this invention, the sequence element above beginning at the end which reads 5' AAAGGT is
15 cloned into an appropriate vector downstream from and operably linked to the promoter. As described elsewhere herein, in preferred embodiments, two, three, four, five, six, seven, or more of the shRNAs encoded by these sequences, optionally, together with other anti-HCV, and/or HBV sequences described herein, are coded into and expressed by a single dsRNA expression vector. In one aspect, each of said multiple stem-loop or shRNA molecules is encoded in a
20 single expression vector within a different expression cassette, each operably linked to a promoter and a terminator, preferably a polymerase III promoter, which may be the same or different. In another aspect, two or more hairpin dsRNA molecules may be expressed from a single promoter, as e.g., a bi-fingered molecule in which a single transcribed RNA strand comprises two such shRNA sequences separated by an unrelated linker sequence. Such
25 constructs, in which a single expression vector provides a mammalian cell with two, three, four, five or more independent dsRNA effector molecules against an HCV and/or HBV target polynucleotide, are particularly desirable for pharmaceutical applications. An alternative means of dsRNA-mediated silencing may be accomplished by preparing shRNAs or duplex dsRNAs corresponding to the identified sequences by chemical synthesis or in vitro expression and

delivering them into a cell in order to achieve inhibition of HCV and/or HBV polynucleotide sequences.

DETAILED DESCRIPTION OF THE INVENTION

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing or transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Since RNA interference acts in a sequence specific manner, the RNAi molecule used as a drug must be specific to its target. Viral genomes are variable to accommodate resistance to changes in the environment. While HBV and HCV are very desirable viral targets for RNAi, the variability and mutability of the viruses and the high rates of transcription of the viruses make HBV and HCV very challenging targets for any therapeutic and/or prophylactic approach. In order to knock down viral genome replication using RNAi there is a need to identify conserved and unique regions in the viral genome. At that same time, it is very important in order to avoid toxicity that any sequences selected for gene silencing be absent from the human genome.

Human Hepatitis B Virus (HBV) Hepatitis B virus belongs to the family of hepadnaviruses. The HBV genome is a relaxed circular, partially double stranded DNA of approximately 3,200 base pairs. There are 4 partially overlapping open reading frames encoding the envelope (pre-S/S), core (precore/core), polymerase, and X proteins. The pre-S/S open reading frames encode the large (L), middle (M), and small (S) surface glycoproteins. The precore/core open reading frame is translated into a precore polypeptide, which is modified into a soluble protein, the hepatitis B e antigen (HBeAg) and the nucleocapsid protein, hepatitis B core antigen. Mutations in the core promoter and precore region have been shown to decrease or abolish HBeAg production. The polymerase protein functions as a reverse transcriptase as well as a DNA polymerase. The X protein is a potent transactivator and may play a role in hepatocarcinogenesis.

The replication cycle of HBV begins with the attachment of the virion to the hepatocyte. Inside the hepatocyte nucleus, synthesis of the plus strand HBV DNA is completed and the viral genome is converted into a covalently closed circular DNA (cccDNA). Most antiviral agents that have been examined so far have little or no effect on cccDNA, which accounts for the rapid reappearance of serum HBV DNA after cessation of antiviral therapy. The aims of treatment of

chronic hepatitis B are to achieve sustained suppression of HBV replication and/or expression of HBV antigens and remission of liver disease.

In GenBank version 132.0 there are more than 4500 HBV sequences and 340 HBV complete genome sequences (317 Human isolates, 22 isolates from other primates and one woodchuck HBV isolate). This variability constitutes a serious challenge for sequence-specific pharmaceutical approaches such as RNAi. In order to identify conserved sequences suitable for RNAi applications, a comparison between all the complete genomes was carried out using a modified version of ClustalW. Two multiple alignment schemes were generated: the first included all 339 HBV complete genome sequences and the second was limited to all Human HBV isolates. The multiple alignment results were parsed and a table that included scores for sequence conservation at each position in the HBV genome was generated. A sliding window search to identify the longest region of sequence conservation larger than 19 nt in length was created. Three major conserved regions were identified and mapped to GenBank accession no.: AF090840, a Human HBV isolate. The conserved HBV sequences were screened against Genbank sequences of both human genomic and cDNA libraries (Human chromosomes database). It was found that 21 nucleotide and longer segments selected as a permuted “window” from within the conserved regions were unique to HBV, i.e. no perfect sequence matches exist between any 21 nt or longer HBV conserved segments and the available sequence databases of human chromosomal and RNA sequences. For human therapeutic purposes, assuring that homologous human sequences are not inadvertently silenced is as important as selecting conserved viral sequences for RNAi.

Human Hepatitis C Virus HCV is a small (40 to 60 nanometers in diameter), enveloped, single-stranded RNA virus of the family Flaviviridae and genus hepacivirus. The genome is approximately 10,000 nucleotides and encodes a single polyprotein of about 3,000 amino acids, which is post-transcriptionally cleaved into 10 polypeptides, including 3 major structural (C, E1, and E2) and multiple non-structural proteins ([NS] NS2 to NS5). The NS proteins include enzymes necessary for protein processing (proteases) and viral replication (RNA polymerase). Because the virus mutates rapidly, changes in the envelope proteins may help it evade the immune system. There are at least 6 major genotypes and more than 90 subtypes of HCV. The different genotypes have different geographic distributions. Genotypes 1a and 1b are the most

common in the United States (about 75 % of cases). Genotypes 2a and 2b (approximately 15%) and 3 (approximately 7%) are less common.

There is little difference in the severity of disease or outcome of patients infected with different genotypes. However, patients with genotypes 2 and 3 are more likely to respond to 5 interferon treatment. The virus replicates at a high rate in the liver and has marked sequence heterogeneity. The main goal of treatment of chronic hepatitis C is to eliminate detectable viral RNA from the blood. Lack of detectable hepatitis C virus RNA from blood six months after completing therapy is known as a sustained response. Studies suggest that a sustained response is equated with a very favorable prognosis and that it may be equivalent to a cure. There may be 10 other more subtle benefits of treatment, such as slowing the progression of liver scarring (fibrosis) in patients who do not achieve a sustained response.

In GenBank version 134.0 there are more than 20,000 HCV sequences and 93 HCV complete genome sequences. A comparison between all the complete genomes was carried out using a modified version of ClustalW. The multiple alignment result was parsed and a table that 15 included scores for sequence conservation at each position in the HCV genome was generated. A sliding window search to identify the longest region of sequence conservation larger than 19 nt in length was created. Three major conserved regions were identified and mapped to GenBank RefSeq (reference sequence) accession no.: NC_004102 this is GenBank annotated HCV complete genome. The three major conserved regions include a portion of the 3' untranslated 20 region of the virus, already described in the literature to be well-conserved among viral isolates. See, e.g., US Patent 5,874,565, "Nucleic Acids Comprising a Highly Conserved Novel 3' Terminal Sequence Element of the Hepatitis C Virus." However, the instant disclosure represents a comprehensive and detailed analysis of these conserved regions to the extent that permitted the discovery and evaluation of multiple short segments suitable for use alone and in 25 combination as a therapeutic for silencing HCV among a diverse patient population. The conserved sequences were screened against Genbank sequences of both human genomic and cDNA libraries (human chromosomes database), and the series of permuted HCV segments greater than 20 bases long with no homology to the human sequence databases were identified.

Non-Homology with Human Sequences

It is equally important to ensure that conserved viral sequences targeted for silencing according to the invention be substantially non-homologous to any naturally occurring, normally functioning, and essential human polynucleotide sequence, so that the dsRNA molecule does not

5 adversely affect the function of any essential naturally occurring mammalian polynucleotide sequence, when used in the methods of this invention. Such naturally occurring functional mammalian polynucleotide sequences include mammalian sequences that encode desired proteins, as well as mammalian sequences that are non-coding, but that provide for essential regulatory sequences in a healthy mammal. Essentially, the RNA molecule useful in this

10 invention must be sufficiently distinct in sequence from any mammalian polynucleotide sequence for which the function is intended to be undisturbed after any of the methods of this invention are performed. Computer algorithms may be used to define the essential lack of homology between the RNA molecule polynucleotide sequence and the normal mammalian sequences.

15 Since the length of a contiguous dsRNA sequence capable of association with and activation of RISC (RNA-induced silencing complex), is generally considered to be 19-27 base pairs, the identified conserved HBV and HCV sequences were compared with both human genomic libraries and, perhaps even more importantly, with human cDNA libraries as described above. Since human cDNA libraries represent expressed sequences that appear in mRNAs, such

20 mRNA sequences would be especially vulnerable to silencing by homologous dsRNA sequences provided to a cell.

Accordingly, the conserved HBV and HCV sequences were compared with human genomic and cDNA sequences. No human cDNA library matches to the HBV or HCV conserved sequences were identified. (Although there were some matches that were ultimately

25 identified as HBV contamination in the cDNA library.) A comparison with human genomic library sequences revealed no match of any sequence of 21 nts or more, one match of 20 nucleotides, and one match of 19 nucleotides. These matches were in non-coding regions, and likely do not appear in mRNA since cognates were not turned up in the cDNA library. Therefore, they are considered unlikely to be a safety risk, but could be excluded if desired.

Conserved sequences from HBV and HCVHBV Conserved Region 1

GAACATGGAGA[A(89%)/G(11%)]CA[T(76%)/C(24%)][C(78%)/A(20%)/T(2%)][A(7
8%)/G(21%)/T(1%)]1CATCAGGA[T(65%)/c(35%)]TCCTAGGACCCCTGCTCGTGTACAG
5 GCGG[G(88%)/t(12%)]GT[T(89%)/G(11%)]TTTCT[T(94%)/C(6%)]GTTGACAA[G(64%)/A
36%]AATCCTCACAAATACC[A(56%)/G(43%)/T(1%)]CAGAGTCTAGACTCGTGGTGGAC
TTCTCTCAATTCTAGGGG[G(92%)/A(5%)/T(3%)]A[A(41%)/G(30%)/T(18%)/C(11%)][C
(90%)/T(10%)]

HBV Conserved Region 2

10 TGGATGTGTCT[G(99%)/A(1%)]CGGCGTTTATCAT

HBV Conserved Region 3

AAGGCCTTCT[A(43%)/G(43%)/C(14%)][T(56%)/A(37%)/C(7%)]GT[A(87%)/C(13
%)]AAC[A(57%)/G(43%)]TA[T(59%)/C(41%)][C(59%)/A(41%)]TG[A(92%)/C(8%)][A(93%
%)/C(7%)]CaTTTACCCCGTTGC[T(54%)/C(46%)][C(92%)/A(8%)]GGCAACGG[C(74%)/T(24
%)][C(A(50%)/T(43%)/c(7%)]GG[T(87%)/C(13%)]CT[G(70%)/C(19%)/T(7%)/A(4%)]TGCCA
15 AGTGTGCTGACGCAACCCCCACTGG[C(48%)/T(38%)/A(14%)]TGGGGCTTGG[C(84
%)/T(16%)][C(84%)/T(12%)/G(4%)]AT[A(47%)/T(23%)/G(17%)/C(13%)]GGCCATC[A(83%
%)/G(17%)][G(92%)/C(8%)]CGCATGCGTGGAACCTT[G(84%)/C(13%)/T(3%)][T(92%)/A(4
%)/C(3%)/G(1%)]G[G(78%)/T(22%)]CTCCTCTGCCGATCCATACTGCGGAACTCCT[A(88
%)
20 /T(9%)/G(1%)/C(1%)]GC[C(57%)/A(35%)/T(6%)/G(2%)]GC[T(92%)/C(7%)/G(1%)]TGTT
T[T(88%)/C(12%)]GCTCGCAGC[C(64%)/A(36%)]GGTCTGG[A(87%)/G(13%)]GC

HBV Conserved Region 4

[C(62%)/T(38%)]ACTGTTCAAGCCTCAAGCTGTGCCTGGGTGGCTT[G(88%)/A
(12%)]GG[G(92%)/A(8%)]CATGGACATTGAC[C(92%)/A(8%)]C[T(65%)/G(35%)]TATAAA
25 GAATTGGAGCT[A(65%)/T(35%)]CTGTGGAGTTACTCTC[G(62%)/T(35%)/A(3%)]TTTT
TGCCTTC[T(92%)/C(8%)]GACTT[C(92%)/T(8%)]TTTCCTTC

HBV Conserved Region 5

[C(69%)/del(31%)]1[G(69%)/del(31%)]A[G(85%)/T(11%)/C(4%)]GCAGG
TCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACG[C(61%)/A(39%)]1AG[A(62%)]

G(38%)]TCTCAATCG[C(88%)/A(12%)]CGCGTCGCAGAACGATCTCAAT[C(92%)/T(8%)]TCGGGAATCT[C(88%)/T(12%)]AATGTTAGTAT

HBV Conserved Region 6

5 TTGG[C(84%)/t(16%)][C(84%)/t(12%)/g(4%)]AT[A(47%)/t(23%)/g(17%)/c(13%)]GG
 CCATC[A(83%)/g(17%)][G(92%)/c(8%)]CGCATGCGTGGAACCTTT[G(84%)/c(13%)/t(3%)]
 [T(92%)/a(4%)/c(3%)/g(1%)]G[G(78%)/t(22%)]CTCCTCTGCCGATCCATACTGCGGAACCT
 CCT[A(88%)/t(9%)/g(1%)/c(1%)]GC[C(57%)/a(35%)/t(6%)/g(2%)]GC[T(92%)/c(7%)/g(1%)]T
 GTTT[T(88%)/c(12%)]GCTCGCAGC[C(64%)/a(36%)]GGTCTGG[A(87%)/g(13%)]GC

10 HBV Conserved Region 7

CTGCCAACTGGAT[C(86%)/T(10%)/A(4%)]CT[C(69%)/T(25%)/A(6%)]CGCGGGA
 CGTCCTTGT[T(75%)/C(25%)]TACGTCCCGTC[G(93%)/A(7%)]GCGCTGAATCC[C(86%)/
 T(7%)/A(7%)]GCGGACGACCC[C(52%)/G(25%)/T(19%)/A(4%)]

HCV Conserved Region 1

15 [A(74%)/G(19%)/T(7%)][G(82%)/A(15%)/T(3%)]ATCACTCCCCGTGAGGAACTA
 CTGTCTTCACGCAGAAAGCGTAGCCATGGCGTTAGTATGAGTGT[C(92%)/T(7%)]G
 TGCAGC[C(89%)/T(10%)]TCCAGG[A(76%)/T(14%)/C(8%)/G(1%)]CCCCCCCTCCCGGGA
 GAGCCATAGTGGTCTGCCAACCGGTGAGTACACCGGAATTGCC[A(90%)/G(9%)]GG
 A[C(78%)/T(16%)/A(5%)]GACCGGGTCCTTCTTGGAT[G(78%)/T(11%)/A(10%)]AACCC
 20 GCTC[A(94%)/T(5%)]ATGCC[T(90%)/C(9%)]GGA[G(91%)/C(4%)/A(4%)]ATTGGGCGTG
 CCCCCCGC[G(85%)/A(14%)]AGAC[T(94%)/C(5%)]GCTAGCCGAGTAG[T(92%)/C(7%)]GT
 TGGGT[C(94%)/T(5%)]GCGAAAGGCCTGTGGTACTGCCTGATAGGGTGCTGCGAGT
 GCCCCGGGAGGTCTCGTAGACCGTGCA[C(62%)/T(30%)/A(8%)]CATGAGCAC[A(50%)/
 G(50%)][A(92%)/C(8%)][A(89%)/T(11%)]TCC[T(92%)/A(5%)/C(3%)]AAACC[T(84%)/C(14
 25 %)/A(2%)]CAAAGAAAAACCAAA[C(84%)/A(16%)]G[T(84%)/A(16%)]AACACCAACCG[
 C(77%)/T(23%)]CGCCCACAGGACGT[C(81%)/T(18%)/A(1%)]AAGTMCCGGG[C(89%)/T(11%)]GG
 [T(80%)/C(20%)]GG[T(80%)/C(17%)/A(3%)]CAGATCGTTGG[T(91%)/C(8%)/G(1%)]GGAGT
 [T(87%)/A(11%)/C(2%)]TAC[C(74%)/T(20%)/G(6%)]TGTTGCCGCGCAGGG
 CCC[C(87%)/T(8%)/A(4%)/G(1%)][A(92%)/C(8%)][G(92%)/A(5%)/C(2%)][G(87%)/A(12%)/
 30 T(1%)]TTGGGTGTGCGCGCGAC[T(78%)/G(13%)/A(7%)/C(2%)]AGGAAGACTTC[C(90%)

/G(5%)/T(5%)]GA[G(90%)/A(10%)]CGGTC[G(79%)/C(12%)/A(8%)/T(1%)]CA[A(86%)/G(14%)])CC[T(88%)/A(6%)C(6%)]CG[T(82%)/C(9%)A(9%)]GG[A(87%)/T(8%)/G(3%)/C(2%)]AG

HCV Conserved Region 2

ATGGC[T(76%)/A(12%)/C(10%)/G(2%)]TGGGATATGATGATGAACTGG[T(81%)/C

5 (19%)]C

Conserved Consensus Sequences presented in SEQ ID format

The following sequences are presented in the format required per the WIPO Standard ST.25 (1998), using the codes provided under 37 CFR 1.821. SEQ ID NO:1 through SEQ ID NO:10 are derived from the HBV genome SEQ ID NO:11 and SEQ ID NO:12 are derived from 10 the HCV genome.

SEQ ID NO:1 HBV

GAACATGGAGArCAyhdCATCAGGAyTCCTAGGACCCCTGCTCGTGTACAGGC
GGkGTkTTCTyCTTGACAArAATCCTCACAAATACCdCAGAGTCTAGACTCGTGGTGA
CTTCTCTCAATTCTAGGGGdAny

15 EQ ID NO:2 HBV

TGGATGTGTCTrCGGCGTTITATCAT

SEQ ID NO:3 HBV

AAGGCCTTCTvhGTmAACArTAymTGmmCCTTACCCC
GTTGCymGGCAACGGye hGGyCTnTGCCAAGTGTGCTGACGCAACCCCCACTGG
20 hTGggGCTTGGybATnGCCATCrsCGCATGCGTGGAA
CCTTbngkCTCCTCTGCCGATCCATACTGCGGAACCTCTnGCnGCbT
GTTTyGCTCGCAGCmGGTCTGGGrGC

SEQ ID NO:4 HBV

yACTGTTCAAGCCTCAAGCTGTGCCTTGGGTGGCTTTrG
25 GrCATGGACATTGACmCkTATAAAGAATTGGAGCTwCTGTGGAGTTACTCTCdTTTT
TGCCTTCyGACTTyTTTCCTTC

SEQ ID NO:5 HBV

CGAbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCTCGCAGACG
mAGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTA
 GTAT

- 5 SEQ ID NO:6 HBV
A_bGCAGGTCCCCTAGAAGAAGAACTCCCTGCCTCGCAGACGmA
GrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTAGTAT
- 10 SEQ ID NO:7 HBV
CAbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCTCGCAGACGm
AGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTAGTAT
- 15 SEQ ID NO:8 HBV
GAbGCAGGTCCCCTAGAAGAAGAACTCCCTGCCTCGCAGACGm
AGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAATCTyAATGTTAGTAT
- 20 SEQ ID NO:9 HBV
 TTGGybATnGCCATCrsCGCATGCGTGGAACCTTbnGk
 CTCCTCTGCCGATCCATACTGCGGAACTCCTnG
CnGCbTGTTTyGCTCGCAGCmGGTCTGGrGC
- 25 SEQ ID NO:10
 HBV CTGCCAACTGGATHCThCGCGGACGTCTTGTyTACG
 TCCC GTCrGCGCTGAATCChGCGGACGACCCn
- 30 SEQ ID NO:11 HCV
 DdATCACTCCCCTGTGAGGAACTACTGTCTTCACGCAGA
 AAGCGTCTAGCCATGGCGTTAGTATGAGTGTyGTGCAGCyTCCAGGn
 CCCCCCCTCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTA
 CACCGGAATTGCCrGGAhGACCGGGTCCTTCTGGATdAACCCGCT
 CwATGCCyGGAvATTGGCGTGCCTTCCGCrAGACyGCTAGCCGAGT
AGyGTTGGGTyGCGAAAGGCCTGTGGTACTGCCTGATAGGGTGCCTGCGAGTGC CC

CGGGAGGTCTCGTAGACCGTGC^AhCATGAGCACrmwTCChAAACChCAAAGAAAAAC
 CAAAmGwAACACCAACCGyCGCC
 CACAGGACGThAAGTTCCC^GGGyGGyGGhCAGATCGTTGGbGGAGThTAC
 bTGTTGCCGCGCAGGGGCCNmvdTTGGGTGTGCGCGACnAGGA

5 AGACTTCbGArCGGTCnCArCChCGhGGnAG

*Double Stranded RNA Gene Silencing/RNAi By “nucleic acid composition” or “nucleotide” composition is meant any one or more compounds in which one or more molecules of phosphoric acid are combined with a carbohydrate (e.g., pentose or hexose) which are in turn combined with bases derived from purine (e.g., adenine) and from pyrimidine (e.g., thymine).

10 Particular naturally occurring nucleic acid molecules include genomic deoxyribonucleic acid (DNA) and host ribonucleic acid (RNA), as well as the several different forms of the latter, e.g., messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Also included are different DNA molecules which are complementary (cDNA) to the different RNA molecules. Synthesized DNA or a hybrid thereof with naturally occurring DNA, as well as DNA/RNA
 15 hybrids, and peptide nucleic acid (PNA) molecules (Gambari, Curr Pharm Des 2001 Nov;7(17):1839-62) can also be used.

It is contemplated that where the desired nucleic acid molecule is RNA, the T (thymine) in the sequences provided herein is substituted with U (uracil). For example, SEQ ID NO:1 through SEQ ID NO:44 are disclosed herein as DNA sequences. It will be obvious to one of ordinary skill in the art that an RNA effector molecule comprising sequences from any of the aforementioned SEQ ID NOs will have T substituted with U.

Nucleic acids typically have a sequence of two or more covalently bonded naturally-occurring or modified deoxyribonucleotides or ribonucleotides. Modified nucleic acids include, e.g., peptide nucleic acids and nucleotides with unnatural bases.

25 By “dsRNA” or “dsRNA effector molecule” is meant a nucleic acid containing a region of two or more nucleotides that are in a double stranded conformation. It is envisioned that the conserved viral sequences of the invention may be utilized in any of the many compositions of “dsRNA effector molecules” known in the art or subsequently developed which act through a dsRNA-mediated gene silencing or RNAi mechanism, including, e.g., “hairpin” or stem-loop
 30 double-stranded RNA effector molecules in which a single RNA strand with self-complementary

sequences is capable of assuming a double-stranded conformation, or duplex dsRNA effector molecules comprising two separate strands of RNA,. In various embodiments, the dsRNA consists entirely of ribonucleotides or consists of a mixture of ribonucleotides and deoxynucleotides, such as the RNA/DNA hybrids disclosed, for example, by WO 00/63364, filed 5 April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. The dsRNA or dsRNA effector molecule may be a single molecule with a region of self-complementarity such that nucleotides in one segment of the molecule base pair with nucleotides in another segment of the molecule. In various embodiments, a dsRNA that consists of a single molecule consists entirely of 10 ribonucleotides or includes a region of ribonucleotides that is complementary to a region of deoxyribonucleotides. Alternatively, the dsRNA may include two different strands that have a region of complementarity to each other. In various embodiments, both strands consist entirely of ribonucleotides, one strand consists entirely of ribonucleotides and one strand consists entirely of deoxyribonucleotides, or one or both strands contain a mixture of ribonucleotides and deoxyribonucleotides. Desirably, the regions of complementarity are at least 70, 80, 90, 95, 98, 15 or 100% complementary to each other and to a target nucleic acid sequence. Desirably, the region of the dsRNA that is present in a double stranded conformation includes at least 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 50, 75, 100, 200, 500, 1000, 2000 or 5000 nucleotides or includes all of the nucleotides in a cDNA or other target nucleic acid sequence being represented in the dsRNA. In some embodiments, the dsRNA does not contain any single stranded regions, 20 such as single stranded ends, or the dsRNA is a hairpin. In other embodiments, the dsRNA has one or more single stranded regions or overhangs. Desirable RNA/DNA hybrids include a DNA strand or region that is an antisense strand or region (e.g, has at least 70, 80, 90, 95, 98, or 100% complementarity to a target nucleic acid) and an RNA strand or region that is a sense strand or region (e.g, has at least 70, 80, 90, 95, 98, or 100% identity to a target nucleic acid), and vice 25 versa. In various embodiments, the RNA/DNA hybrid is made in vitro using enzymatic or chemical synthetic methods such as those described herein or those described in WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. In other embodiments, a DNA strand synthesized in vitro is complexed with an RNA strand made in vivo or in vitro before, after, or concurrent with the transformation of the DNA strand into the cell. In yet other 30 embodiments, the dsRNA is a single circular nucleic acid containing a sense and an antisense region, or the dsRNA includes a circular nucleic acid and either a second circular nucleic acid or

a linear nucleic acid (see, for example, WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999.) Exemplary circular nucleic acids include lariat structures in which the free 5' phosphoryl group of a nucleotide becomes linked to the 2' hydroxyl group of another nucleotide in a loop back fashion.

- 5 In other embodiments, the dsRNA includes one or more modified nucleotides in which the 2' position in the sugar contains a halogen (such as fluorine group) or contains an alkoxy group (such as a methoxy group) which increases the half-life of the dsRNA in vitro or in vivo compared to the corresponding dsRNA in which the corresponding 2' position contains a hydrogen or an hydroxyl group. In yet other embodiments, the dsRNA includes one or more
- 10 linkages between adjacent nucleotides other than a naturally-occurring phosphodiester linkage. Examples of such linkages include phosphoramido, phosphorothioate, and phosphorodithioate linkages. The dsRNAs may also be chemically modified nucleic acid molecules as taught in U.S. Patent No. 6,673,661. In other embodiments, the dsRNA contains one or two capped strands, as disclosed, for example, by WO 00/63364, filed April 19, 2000, or U.S.S.N.
- 15 60/130,377, filed April 21, 1999. In other embodiments, the dsRNA contains coding sequence or non-coding sequence, for example, a regulatory sequence (e.g., a transcription factor binding site, a promoter, or a 5' or 3' untranslated region (UTR) of an mRNA). Additionally, the dsRNA can be any of the at least partially dsRNA molecules disclosed in WO 00/63364, filed April 19, 2000 (see, for example, pages 8-22), as well as any of the dsRNA molecules described in US
- 20 Provisional Application 60/399,998 filed July 31, 2002, and PCT/US2003/024028, filed 31-Jul-2003; and US Provisional Application 60/419,532 filed October 18, 2002, and PCT/US2003/033466, filed 20-Oct-2003, the teaching of which is hereby incorporated by reference. Any of the dsRNAs may be expressed in vitro or in vivo using the methods described herein or standard methods, such as those described in WO 00/63364, filed April 19, 2000 (see, for example, pages 16-22). In some preferred embodiments, multiple anti-HBV and/or anti-
- 25 HCV dsRNA effector molecules of the invention are transcribed in a mammalian cell from one or more expression constructs each comprising multiple polymerase III promoter expression cassettes as described in more detail in US 60/603622; US 60/629942; and PCT/US05/29976 filed 23-August-2005; “Multiple Polymerase III Promoter Expression Constructs”; the teaching of which is incorporated by reference.
- 30

dsRNA “Hairpin” Constructs or dsRNA “Hairpin” Expression Vectors: Constructs encoding a unimolecular hairpin dsRNA are more desirable for some applications than constructs encoding duplex dsRNA (i.e., dsRNA composed of one RNA molecule with a sense region and a separate RNA molecule with an antisense region) because the single-stranded RNA with inverted repeat sequences more efficiently forms a dsRNA hairpin structure. This greater efficiency is due in part to the occurrence of transcriptional interference arising in vectors containing converging promoters that generate duplex dsRNA. Transcriptional interference results in the incomplete synthesis of each RNA strand thereby reducing the number of complete sense and antisense strands that can base-pair with each other and form duplexes. Transcriptional interference can be overcome, if desired, through the use of (i) a two vector system in which one vector encodes the sense RNA and the second vector encodes the antisense RNA, (ii) a bicistronic vector in which the individual strands are encoded by the same plasmid but through the use of separate cistrons, or (iii) a single promoter vector that encodes a hairpin dsRNA, i.e., an RNA in which the sense and antisense sequences are encoded within the same RNA molecule.

Hairpin-expressing vectors have some advantages relative to the duplex vectors. For example, in vectors that encode a duplex RNA, the RNA strands need to find and base-pair with their complementary counterparts soon after transcription. If this hybridization does not happen, the individual RNA strands diffuse away from the transcription template and the local concentration of sense strands with respect to antisense strands is decreased. This effect is greater for RNA that is transcribed intracellularly compared to RNA transcribed *in vitro* due to the lower levels of template per cell. Moreover, RNA folds by nearest neighbor rules, resulting in RNA molecules that are folded co-transcriptionally (i.e., folded as they are transcribed). Some percentage of completed RNA transcripts is therefore unavailable for base-pairing with a complementary second RNA because of intra-molecular base-pairing in these molecules. The percentage of such unavailable molecules increases with time following their transcription. These molecules may never form a duplex because they are already in a stably folded structure. In a hairpin RNA, an RNA sequence is always in close physical proximity to its complementary RNA. Since RNA structure is not static, as the RNA transiently unfolds, its complementary sequence is immediately available and can participate in base-pairing because it is so close. Once formed, the hairpin structure is predicted to be more stable than the original non-hairpin structure. Especially desirable are, e.g., “forced” hairpin constructs, partial hairpins capable of being

extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002; and PCT/US2003/024028, “Double Stranded RNA Structures and Constructs and Methods for Generating and Using the Same,” filed 31-Jul-2003; as well as the “udderly” structured hairpins, hairpins with mismatched regions, and multiepitope constructs
5 as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, “Double-Stranded RNA Structures and Constructs, and Methods for Generating and Using the Same,” filed 20-Oct-2003.

By “short dsRNA” is meant a dsRNA that has about 200, 100, 75, 50, 45, 40, 35, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20 or 19 contiguous nucleotides in length that are in a double stranded conformation. Desirably, the short dsRNA comprises a double-stranded region of at least 19 contiguous basepairs in length identical/complementary to a target sequence to be inhibited. In desirable embodiments, the double stranded region is between 19 to 50, 19 to 40, 19 to 30, 19 to 25, 20 to 25, 21 to 23, 25 to 30, or 30 to 40 contiguous basepairs in length, inclusive. In some embodiments, the short dsRNA is between 30 to 50, 50 to 100, 100 to 200,
15 200 to 300, 400 to 500, 500 to 700, 700 to 1000, 1000 to 2000, or 2000 to 5000 nucleotides in length, inclusive and has a double stranded region that is between 38 and 60 contiguous basepairs in length, inclusive. In one embodiment, the short dsRNA is completely double stranded. In some embodiments, the short dsRNA is between 11 and 30 nucleotides in length, and the entire dsRNA is double stranded. In other embodiments, the short dsRNA has one or
20 two single stranded regions. In some embodiments, the short dsRNA is a “shRNA” or “short-hairpin RNA” or “shRNA effector molecule” or “dsRNA hairpin”, meaning an RNA molecule of less than approximately 400 to 500 nucleotides (nt) in length, preferably less than 100 to 200 nt in length, in which at least one stretch of at least about 15 to 100 nucleotides (preferably 17 to 50 nt; more preferably 19 to 29 nt) is base paired with a complementary sequence located on the
25 same RNA molecule, and where said sequence and complementary sequence are separated by an unpaired region of at least about 4 to 7 nucleotides (preferably about 9 to about 15 nucleotides) which forms a single-stranded loop above the stem structure created by the two regions of base complementarity. The shRNA molecules comprise at least one stem-loop structure comprising a double-stranded stem region of about 17 to about 100 bp; about 17 to about 50 bp; about 40 to
30 about 100 bp; about 18 to about 40 bp; or from about 19 to about 29 bp; homologous and complementary to a target sequence to be inhibited; and an unpaired loop region of at least about

4 to 7 nucleotides; preferably about 9 to about 15 nucleotides, which forms a single-stranded loop above the stem structure created by the two regions of base complementarity. Included shRNAs are dual or bi-finger (i.e., having two stem-loop structures) and multi-finger hairpin dsRNAs (having multiple stem-loop structures), in which the RNA molecule comprises two or 5 more of such stem-loop structures separated by single-stranded spacer regions. In some embodiments, an expression construct may be used to express one or more of such shRNA molecules in a mammalian cell, including multiple copies of the same, and/or one or more, including multiple different, short hairpin RNA molecules. Short hairpin RNA molecules considered to be the “same” as each other are those that comprise only the same double-stranded 10 sequence, and short hairpin RNA molecules considered to be “different” from each other will comprise different double-stranded sequences, regardless of whether the sequences to be targeted by each different double-stranded sequence are within the same, or a different gene, such as, e.g., sequences of a promoter region and of a transcribed region (mRNA) of the same gene, or sequences of two different genes.

15 In particular embodiments, the short dsRNA binds PKR or another protein in a dsRNA-mediated stress response pathway. Desirably, such a short dsRNA inhibits the dimerization and activation of PKR by at least 20, 40, 60, 80, 90, or 100%. In some desirable embodiments, the short dsRNA inhibits the binding of a long dsRNA to PKR or another component of a dsRNA-mediated stress response pathway by at least 20, 40, 60, 80, 90, or 100%. See also the teaching 20 of USSN 10/425,006, filed 28-Apr-2003, “Methods of Silencing Genes Without Inducing Toxicity”, Pachuk, as to utilization of short dsRNAs in conjunction with other dsRNAs to avoid dsRNA-mediated toxicity. The applicants have demonstrated, however, that dsRNA molecules, even long dsRNA molecules, are in general unlikely to evoke a significant dsRNA stress 25 response, including a PKR or interferon or “panic” response, if they are expressed intracellularly in the mammalian (or other vertebrate) cell in which the RNAi effect is desired. See, e.g., US 2002/0132257, “Use of post-transcriptional gene silencing for identifying nucleic acid sequences that modulate the function of a cell”. Accordingly, such “expressed interfering RNA molecules” or “eiRNA” molecules and “eiRNA expression constructs”, i.e., dsRNA molecules (or the corresponding dsRNA expression constructs) expressed intracellularly or endogenously in vivo 30 within the mammalian cell in which dsRNA gene silencing or RNAi is induced, are preferred in some aspects of the invention.

By “at least 19 contiguous base pair nucleotide sequence” is meant that a nucleotide sequence can start at any nucleotide within one of the disclosed sequences, so long as the start site is capable of producing a polynucleotide of at least 19 contiguous base pairs. For example, an at least 19 contiguous base pair nucleotide sequence can comprise nucleotide 1 through 5 nucleotide 19, nucleotide 2 through nucleotide 20, nucleotide 3 through nucleotide 21, and so forth to produce a 19mer. Thus, a 20mer can comprise nucleotide 1 through nucleotide 20, nucleotide 2 through nucleotide 21, nucleotide 3 through nucleotide 22, and so forth. Similar sequences above 20 contiguous nucleotides, e.g., 21, 22, 23, 24, 25, 26, 27, etc. selected from within the conserved sequences are envisioned. Such a sequence of at least 19 contiguous 10 nucleotides (in double-stranded conformation with its complement) is “an at least 19 contiguous base pair sequence” and may be present as a duplex dsRNA, within a dsRNA hairpin, or encoded in a dsRNA expression construct.

By “expression vector” is meant any double stranded DNA or double stranded RNA designed to transcribe an RNA, e.g., a construct that contains at least one promoter operably 15 linked to a downstream gene or coding region of interest (e.g., a cDNA or genomic DNA fragment that encodes a protein, or any RNA of interest, optionally, e.g., operatively linked to sequence lying outside a coding region, an antisense RNA coding region, a dsRNA coding region, or RNA sequences lying outside a coding region). Transfection or transformation of the expression vector into a recipient cell allows the cell to express RNA or protein encoded by the 20 expression vector. An expression vector may be a genetically engineered plasmid, virus, or artificial chromosome derived from, for example, a bacteriophage, adenovirus, retrovirus, poxvirus, or herpesvirus.

By an “expression construct” is meant any double-stranded DNA or double-stranded RNA designed to transcribe an RNA, e.g., a construct that contains at least one promoter 25 operably linked to a downstream gene or coding region of interest (e.g., a cDNA or genomic DNA fragment that encodes a protein, or any RNA of interest). Transfection or transformation of the expression construct into a recipient cell allows the cell to express RNA or protein encoded by the expression construct. An expression construct may be a genetically engineered plasmid, virus, or artificial chromosome derived from, for example, a bacteriophage, adenovirus, 30 retrovirus, poxvirus, or herpesvirus. An expression construct does not have to be replicable in a living cell, but may be made synthetically. An expression construct or expression vector

engineered to express a double-stranded RNA effector molecule or dsRNA molecule is a “dsRNA expression construct” or “dsRNA expression vector”.

In one embodiment of the invention, a recombinant expression vector or expression construct is engineered to express multiple, e.g., three, four, five or more short hairpin dsRNA effector molecules, each expressed from a different expression cassette comprising a polymerase III promoter, one or more, including all of which, may be different from the others. In one aspect of the invention, a recombinant expression vector transcribing three, four, five or more different shRNA molecules (each comprising a double-stranded “stem” region comprising at least 19 contiguous basepairs from/complementary to a conserved HBV and/or HCV sequence) is used to inhibit replication of hepatitis B virus (HBV) and/or hepatitis C virus (HCV). In one embodiment, each shRNA molecule is expressed under the control of a polymerase III promoter, e.g., 7SK, H1, and U6, which may be the same or different. Such dsRNA expression constructs comprising multiple polymerase III expression cassettes are described in greater detail in PCT/US05/29976, “Multiple Polymerase III Promoter Expression Constructs”, the teaching of which is hereby incorporated by reference. In one aspect, a recombinant expression vector or expression construct of the invention may express one or more bi-fingered or multi-fingered dsRNA hairpin molecules from one or more polymerase III promoter-driven transcription units as well as one or more single hairpin dsRNA molecules from one or more polymerase III promoter-driven transcription units. It will be understood that in any of said expression constructs transcribing a hairpin dsRNA from a polymerase III promoter, the hairpin dsRNA may be a single hairpin dsRNA or a bi-fingered, or multi-fingered dsRNA hairpin as described in WO2004/035765, published 29-Apr-2004, or a partial or forced hairpin structure as described in WO2004/011624, published 5-Feb-2004, the teaching of which is incorporated herein by reference.

By “operably linked” is meant that a nucleic acid sequence or molecule and one or more regulatory sequences (e.g., a promoter, enhancer, repressor, terminator) are connected in such a way as to permit transcription of an RNA molecule, e.g., a single-stranded RNA molecule such as a sense, antisense, a dsRNA hairpin, or an mRNA, or permit expression and translation and/or secretion of the product (i.e., a polypeptide) of the nucleic acid molecule when the appropriate molecules are bound to the regulatory sequences.

By a “promoter” is meant a nucleic acid sequence sufficient to direct transcription of a covalently linked nucleic acid molecule. Also included in this definition are those transcription control elements (e.g., enhancers) that are sufficient to render promoter-dependent gene expression controllable in a cell type-specific, tissue-specific, or temporal-specific manner, or
5 that are inducible by external signals or agents; such elements, which are well-known to skilled artisans, may be found in a 5' or 3' region of a gene or within an intron. See, e.g., published U.S. Patent Application No. 2005/0130184 A1, 16-June-2005, Xu et al., directed to modified polymerase III promoters which utilize polymerase II enhancer elements, as well as Published U.S. Patent Application No. 2005/0130919 A1, 16-June-2005, Xu et al., directed to regulatable 10 polymerase III and polymerase II promoters, the teaching of which is hereby incorporated by reference. Desirably a promoter is operably linked to a nucleic acid sequence, for example, a cDNA or a gene sequence, or a sequence encoding a dsRNA, e.g., a shRNA, in such a way as to permit expression of the nucleic acid sequence.

The RNA molecule according to this invention may be delivered to the mammalian cell
15 or extracellular pathogen present in the mammalian cell in the composition as a dsRNA effector molecule or partially double stranded RNA sequence, or RNA/DNA hybrid, which was made in vitro by conventional enzymatic synthetic methods using, for example, the bacteriophage T7, T3 or SP6 RNA polymerases according to the conventional methods described by such texts as the Promega Protocols and Applications Guide, (3rd ed. 1996), eds. Doyle, ISBN No. 1 57
20 Alternatively these molecules may be made by chemical synthetic methods in vitro [see, e.g., Q. Xu et al., Nucleic Acids Res., 24(18):3643-4 (Sept. 1996); N. Naryshkin et al., Bioorg. Khim., 22(9):691-8 (Sept. 1996); J. A. Grasby et al., Nucleic Acids Res., 21(19):4444-50 (Sept 1993); C. Chaix et al., Nucleic Acids Res. 17:7381-93 (1989); S.H. Chou et al., Biochem., 28(6):2422-35 (Mar. 1989); O. Odal el al., Nucleic Acids Symp. Ser., 21:105-6 (1989); N.A. Naryshkin et al.,
25 Bioorg. Khim, 22(9):691-8 (Sept. 1996); S. Sun et al., RNA, 3(11):1352-1363 (Nov. 1997); X. Zhang et al., Nucleic Acids Res., 25(20):3980-3 (Oct. 1997); S. M. Grvaznov el al., Nucleic Acids Res., 2-6 (18):4160-7 (Sept. 1998); M. Kadokura et al., Nucleic Acids Symp. Ser., 37:77-8 (1997); A. Davison et al., Biomed. Pept. Proteins. Nucleic Acids, 2(I):1-6 (1996); and A. V. Mudrakovskaia et al., Bioorg. Khirn., 17(6):819-22 (Jun. 1991)].

30 Still alternatively, the RNA molecule of this invention can be made in a recombinant microorganism, e.g., bacteria and yeast or in a recombinant host cell, e.g., mammalian cells, and

isolated from the cultures thereof by conventional techniques. See, e.g., the techniques described in Sambrook et al, MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, which is exemplary of laboratory manuals that detail these techniques, and the techniques described in US Patent Nos. 5,824,538; 5,877,159; and 5,643,771, incorporated herein by reference.

Such RNA molecules prepared or synthesized *in vitro* may be directly delivered to the mammalian cell or to the mammal as they are made *in vitro*. The references above provide one of skill in the art with the techniques necessary to produce any of the following specific embodiments, given the teachings provided herein. Therefore, in one embodiment, the “agent” of the composition is a duplex (i.e., it is made up of two strands), either complete or partially double stranded RNA.

In another embodiment, the agent is a single stranded RNA sense strand. In another embodiment, the agent of the composition is a single stranded RNA anti-sense strand.

Preferably the single stranded RNA sense or anti-sense strand forms a hairpin at one or both termini. Desirably, the single stranded RNA sense or anti- sense strand forms a hairpin at some intermediate portion between the termini. Such a single stranded RNA sense or anti-sense strand may also be designed to fold back upon itself to become partially double stranded *in vitro* or *in vivo*. Yet another embodiment of an extant RNA molecule as the effective agent used in the compositions is a single stranded RNA sequence comprising both a sense polynucleotide sequence and an antisense polynucleotide sequence, optionally separated by a non-base paired polynucleotide sequence. Preferably, this single stranded RNA sequence has the ability to become double-stranded once it is in the cell, or *in vitro* during its synthesis. In desirable embodiments, a sequence of at least about 19 to 29 contiguous basepairs will assume a double-stranded conformation. In desirable embodiments, the double-stranded region will include an at least about 19 contiguous basepair sequence identical/complementary to a target nucleotide sequence to be downregulated or inhibited.

Still another embodiment of this invention is an RNA/DNA hybrid as described above.

Still another embodiment of the synthetic RNA molecule is a circular RNA molecule that optionally forms a rod structure [see, e.g., K-S. Wang et al., Nature 323:508-514 (1986)] or is partially double-stranded, and can be prepared according to the techniques described in S. Wang et al., Nucleic Acids Res., 22(12):2326-33 (June 1994); Y. Matsumoto et al., Proc. Natl. Acad.

Sci, USA, 87(19):7628-32 (Oct. 1990); E. Ford & M. Ares, Proc. Natl. Acad. Sci. USA 91(8):3117-21 (Apr. 1994); M. Tsagris et al., Nucleic Acids Res., 19 7):1605-12 (Apr. 1991); S. Braun et al., Nucleic Acids Res. 24(21):4152-7 (Nov. 1996); Z. Pasman et al., RNA, 2(6):603-10 (Jun. 1996); P. G. Zaphiropoulos, Proc. Natl. Acad. Sci., USA, 93(13):6536-41 (Jun. 1996); D.

- 5 Beaudry et al., Nucleic Acids Res., 23(15):3064-6 (Aug. 1995), all incorporated herein by reference. Still another agent is a double-stranded molecule comprised of RNA and DNA present on separate strands, or interspersed on the same strand.

Alternatively, the RNA molecule may be formed in vivo and thus delivered by a “delivery agent” which generates such a partially double-stranded RNA molecule in vivo after 10 delivery of the agent to the mammalian cell or to the mammal. Thus, the agent which forms the composition of this invention is, in one embodiment, a double stranded DNA molecule “encoding” one of the above-described RNA molecules, e.g., a dsRNA expression vector or expression construct. The DNA agent provides the nucleotide sequence which is transcribed within the cell to become a double stranded RNA. In another embodiment, the DNA sequence 15 provides a deoxyribonucleotide sequence which within the cell is transcribed into the above-described single stranded RNA sense or anti-sense strand, which optionally forms a hairpin at one or both termini or folds back upon itself to become partially double stranded. The DNA molecule which is the delivery agent of the composition can provide a single stranded RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide 20 sequence, optionally separated by a nonbase paired polynucleotide sequence, and wherein the single stranded RNA sequence has the ability to become double-stranded. Alternatively, the DNA molecule which is the delivery agent provides for the transcription of the above-described circular RNA molecule that optionally forms a rod structure or partial double strand in vivo. The DNA molecule may also provide for the in vivo production of an RNA/DNA hybrid as described 25 above, or a duplex containing one RNA strand and one DNA strand. These various DNA molecules may be designed by resort to conventional techniques such as those described in Sambrook, cited above or in the Promega reference, cited above.

A latter delivery agent of the present invention, which enables the formation in the mammalian cell of any of the above-described RNA molecules, can be a DNA single stranded or 30 double stranded plasmid or vector. Expression vectors designed to produce RNAs as described herein in vitro or in vivo may contain sequences under the control of any RNA polymerase,

including mitochondria! RNA polymerase, RNA pol I, RNA pol II, and RNA pol III, and viral polymerases, and bacteriophage polymerases such as T7 and Sp6. Desirably, expression vectors designed for in vivo expression of dsRNA effector molecules within a mammalian cell may be designed to utilize an endogenous mammalian polymerase such as an RNA polymerase I, RNA 5 polymerase II, RNA polymerase III, and/or a mitochondrial polymerase. Expression vectors utilizing cognate promoter(s), e.g., polymerase III promoters such as U6, H1, or 7SK, in order to effect transcription by RNA polymerase III can readily be designed. Preferred for expression of short RNA molecules less than about 400 to 500 nucleotides in length are RNA polymerase III promoters. In some aspects, an “RNA polymerase III promoter” or “RNA pol III promoter” or 10 “polymerase III promoter” or “pol III promoter” is preferred, meaning any invertebrate, vertebrate, or mammalian promoter, e.g., human, murine, porcine, bovine, primate, simian, etc. that, in its native context in a cell, associates or interacts with RNA polymerase III to transcribe its operably linked gene, or any variant thereof, natural or engineered, that will interact in a selected host cell with an RNA polymerase III to transcribe an operably linked nucleic acid 15 sequence. Preferred in some applications are the Type III RNA pol III promoters including U6, H1, 7SK, and MRP which exist in the 5' flanking region, include TATA boxes, and lack internal promoter sequences. One reason RNA Pol III promoters are especially desirable for expression of small engineered RNA transcripts is that RNA Pol III termination, unlike RNA polymerase II termination, occurs efficiently and precisely at a short run of thymine residues in the DNA 20 coding strand, without other protein factors, T4 and T5 being the shortest Pol III termination signals in yeast and mammals, with oligo (dT) terminators longer than T5 being very rare in mammals. Accordingly, the multiple polymerase III promoter expression constructs of the invention will include an appropriate oligo (dT) termination signal, i.e., a sequence of 4, 5, 6 or more Ts, operably linked 3' to each RNA Pol III promoter in the DNA coding strand.

25 These vectors can be used to transcribe the desired RNA molecule in the cell according to this invention. Vectors may be desirably designed to utilize an endogenous mitochondrial RNA polymerase (e.g., human mitochondrial RNA polymerase, in which case such vectors may utilize the corresponding human mitochondrial promoter). Mitochondria! polymerases may be used to generate capped (through expression of a capping enzyme) or uncapped messages in vivo. RNA 30 pol I, RNA pol II, and RNA pol III transcripts may also be generated in vivo. Such RNAs may be capped or not, and if desired, cytoplasmic capping may be accomplished by various means

including use of a capping enzyme such as a vaccinia capping enzyme or an alphavirus capping enzyme. However, all pol II transcripts are capped. The DNA vector is designed to contain one of the promoters or multiple promoters in combination (mitochondrial, RNA pol I, pol II, or pol III, or viral, bacterial or bacteriophage promoters along with the cognate polymerases).

- 5 Preferably, where the promoter is RNA pol II, the sequence encoding the RNA molecule has an open reading frame greater than about 300 nts and must follow the rules of design to prevent nonsense-mediated degradation in the nucleus. Such plasmids or vectors can include plasmid sequences from bacteria, viruses or phages.

Such vectors include chromosomal, episomal and virus-derived vectors, e.g., vectors
10 derived from bacterial plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses, vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids and phagemids.

Thus, one exemplary vector is a single or double-stranded phage vector. Another
exemplary vector is a single or double-stranded RNA or DNA viral vector. Such vectors may be
15 introduced into cells as polynucleotides, preferably DNA, by well known techniques for
introducing DNA and RNA into cells. The vectors, in the case of phage and viral vectors may
also be and preferably are introduced into cells as packaged or encapsidated virus by well known
techniques for infection and transduction. Viral vectors may be replication competent or
replication defective. In the latter case, viral propagation generally occurs only in
20 complementing host cells.

In another embodiment the delivery agent comprises more than a single DNA or RNA
plasmid or vector. As one example, a first DNA plasmid can provide a single stranded RNA
sense polynucleotide sequence as described above, and a second DNA plasmid can provide a
single stranded RNA anti-sense polynucleotide sequence as described above, wherein the sense
25 and anti-sense RNA sequences have the ability to base-pair and become double-stranded. Such
plasmid(s) can comprise other conventional plasmid sequences, e.g., bacterial sequences such as
the well-known sequences used to construct plasmids and vectors for recombinant expression of
a protein. However, it is desirable that the sequences which enable protein expression, e.g.,
Kozak regions, etc., are not included in these plasmid structures.

30 The vectors designed to produce dsRNAs of the invention may desirably be designed to
generate two or more, including a number of different dsRNAs homologous and complementary

to a target sequence. This approach is desirable in that a single vector may produce many, independently operative dsRNAs rather than a single dsRNA molecule from a single transcription unit and by producing a multiplicity of different dsRNAs, it is possible to self select for optimum effectiveness. Various means may be employed to achieve this, including

- 5 autocatalytic sequences as well as sequences for cleavage to create random and/or predetermined splice sites.

Other delivery agents for providing the information necessary for formation of the above-described desired RNA molecules in the mammalian cell include live, attenuated or killed, inactivated recombinant bacteria which are designed to contain the sequences necessary for the
10 required RNA molecules of this invention. Such recombinant bacterial cells, fungal cells and the like can be prepared by using conventional techniques such as described in US Patent Nos. 5,824,538; 5,877,159; and 5,643,771, incorporated herein by reference. Microorganisms useful in preparing these delivery agents include those listed in the above cited reference, including, without limitation, Escherichia coli, Bacillus subtilis, Salmonella typhimurium, and
15 various species of Pseudomonas, Streptomyces, and Staphylococcus.

Still other delivery agents for providing the information necessary for formation of the desired, above-described RNA molecules in the mammalian cell include live, attenuated or killed, inactivated viruses, and particularly recombinant viruses carrying the required RNA polynucleotide sequence discussed above. Such viruses may be designed similarly to
20 recombinant viruses presently used to deliver genes to cells for gene therapy and the like, but preferably do not have the ability to express a protein or functional fragment of a protein. Among useful viruses or viral sequences which may be manipulated to provide the required RNA molecule to the mammalian cell *in vivo* are, without limitation, alphavirus, adenovirus, adeno associated virus, baculoviruses, delta virus, pox viruses, hepatitis viruses, herpes viruses,
25 papova viruses (such as SV40), poliovirus, pseudorabies viruses, retroviruses, lentiviruses, vaccinia viruses, positive and negative stranded RNA viruses, viroids, and virusoids, or portions thereof. These various viral delivery agents may be designed by applying conventional techniques such as described in M. Di Nocla et al., Cancer Gene Ther., 5(6):350-6 (1998), among others, with the teachings of the present invention.

The term “in vivo” is intended to include any system wherein the cellular DNA or RNA replication machinery is intact, preferably within intact living cells, including tissue culture systems, tissue explants, and within single cell or multicellular living organisms.

By “multiple sequitope dsRNA” or “multisequitope dsRNA” or “multiple epitope dsRNA” is meant an RNA molecule that has segments derived from multiple target nucleic acids or that has non-contiguous segments from the same target nucleic acid. For example, the multiple sequitope dsRNA may have segments derived from (i) sequences representing multiple genes of a single organism; (ii) sequences representing one or more genes from a variety of different organisms; and/or (iii) sequences representing different regions of a particular gene (e.g., one or more sequences from a promoter and one or more sequences from an mRNA). Desirably, each segment has substantial sequence identity to the corresponding region of a target nucleic acid. In various desirable embodiments, a segment with substantial sequence identity to the target nucleic acid is at least 19, 20, 21, 22, 23, 24, 25, 26, 27, 30, 40, 50, 100, 200, 500, 750, or more basepairs in length. In desirable embodiments, the multiple epitope dsRNA inhibits the expression of at least 2, 4, 6, 8, 10, 15, 20, or more target genes by at least 20, 40, 60, 80, 90, 95, or 100%. In some embodiments, the multiple epitope dsRNA has non-contiguous segments from the same target gene or from the same target polynucleotide that may or may not be in the naturally occurring 5’ to 3’ order of the segments, and the dsRNA inhibits the expression of the target nucleic acid by at least 50, 100, 200, 500, or 1000% more than a dsRNA with only one of the segments.

By “sequitope” is meant a contiguous sequence of double-stranded polyribonucleotides that can associate with and activate RISC (RNA-induced silencing complex), usually a contiguous sequence of between 19 and 27 basepairs, inclusive. Sequences comprising at least one sequitope from within one or more of the conserved HBV and/or HCV nucleotide sequences identified here may be utilized for dsRNA mediated gene silencing as taught herein.

Multiple-epitope/ multiple-sequitope dsRNAs the advantages of a multiple-epitope or multisequitope double-stranded RNA approach as taught in USSN 60/419,532, filed 18-Oct-2002 and PCT/US2003/033466, filed 20-Oct-2003, are applicable to utilization of the conserved HBV and/or HCV sequences of the invention. Because a singular species of dsRNA can simultaneously silence many target genes (e.g., genes from multiple pathogens, multiple genes or sequences from a single pathogen, or genes associated with multiple diseases), a multiple epitope

dsRNA can be used for many different indications in the same subject or used for a subset of indications in one subject and another subset of indications in another subject. For such applications, the ability to express long dsRNA molecules (e.g., dsRNA molecules with sequences from multiple genes) without invoking the dsRNA stress response is highly desirable.

- 5 For example, by using a series of sequences, each, e.g., as short as 19-21 nucleotides, desirably 100 to 600 nucleotides, or easily up to 1, 2, 3, 4, 5, or more kilobases such that the total length of such sequences is within the maximum capacity of the selected plasmid (e.g., 20 kilobases in length), a single such pharmaceutical composition can provide protection against a large number of pathogens and/or toxins at a relatively low cost and low toxicity, e.g., HBV, HCV, HIV, etc.
- 10 The use of multiple epitopes or sequitopes derived from one or more genes and/or different overlapping and/or non-contiguous sequences of the same polynucleotide or gene from multiple strains and/or variants of a highly variable or rapidly mutating pathogen such as HBV and/or HCV can also be very advantageous. For example, a singular dsRNA species that recognizes and targets multiple strains and/or variants of HBV and/or HCV can be used as a
- 15 universal treatment or vaccine for the various strains/variants of HBV and/or HCV.

The ability to silence multiple genes of a particular pathogen such as HBV and/or HCV prevents the selection of, in this case, HBV and/or HCV “escape mutants.” In contrast, typical small molecule treatment or vaccine therapy that only targets one gene or protein results in the selection of pathogens that have sustained mutations in the target gene or protein and the 20 pathogen thus becomes resistant to the therapy. By simultaneously targeting a number of genes or sequences of the pathogen and/or extensive regions of the pathogen using the multiple epitope approach of the present invention, the emergence of such “escape mutants” is effectively precluded.

For example, it is considered particularly advantageous to include a mixture of sequences 25 from both HCV SEQ ID NO:11 and SEQ ID NO:12, and SEQ ID NO: 27, i.e., one or more sequences (e.g, each at least 19, 20, 21, 22, 23, 24, 25, 26, 27 to 29 contiguous nucleotides) from HCV SEQ ID NO:11 together with one or more sequences (e.g, each at least 19, 20, 21, 22, 23, 24, 25, 26, 27 to 29 contiguous nucleotides) from HCV SEQ ID NO:12 and from SEQ ID NO: 30 27 , either in a single dsRNA molecule, an admixture of dsRNA molecules, or through concomitant administration of such molecules to a patient (or by administering one or more dsRNA expression constructs which produce such dsRNA molecules intracellularly), in order to

decrease the ability of the virus to generate viable escape mutants. Similarly, it would be advantageous to provide a mixture of dsRNA molecules comprising a number of the conserved HBV sequences, in some cases in combination with one or more of the conserved HCV sequences of the invention.

- 5 Similarly, it may be desirable to use sequences from two or more of HBV SEQ ID NO:1, SEQ ID NO:2, AND SEQ ID NO:3, either in a single dsRNA construct, an admixture of constructs, or through concomitant administration of such constructs (or dsRNA expression constructs which produce such dsRNA molecules) to a patient. SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 map to the HBV surface antigen genes. Due to the overlapping nature of the
- 10 HBV mRNAs, the following mRNAs would be targeted by one of more of these sequences: Surface Ag (sAg) mRNAs, precore, core and polymerase mRNAs. However, since sAg mRNAs are the most abundant, it is more likely that these mRNAs will be targeted if the gene-silencing machinery is saturable. It is possible, however, that all listed mRNAs will be targeted.
- 15 Reduction of surface Ag is desirable for several reasons: a) surface Ag is needed for assembly of infectious virions; b) overexpression of Surface Ag during infection is thought to contribute to immune anergy that occurs during chronic HBV infection; and c) the expression of HBVsAg in the livers of infected individuals (even in the absence of virus, i.e., from integrated sAg sequences into the host genome) induces hepatitis. Therefore, reduction of sAg is likely to decrease viral titers, overcome immune anergy and decrease/prevent hepatitis.
- 20 HBV SEQ ID NO:4 maps to the unique region of precore and core and will target these mRNAs specifically. Core protein is needed to make functional virions and so down regulation of this mRNA is predicted to decrease viral titers. There should be no competition of these effector RNAs for surface, polymerase or X mRNAs.
- 25 HBV SEQ ID NO:5 through SEQ ID NO:8 map to the polymerase gene. Effector RNAs are predicted to target only precore/core and polymerase transcripts. There should be no competition with sAg or X mRNAs. Polymerase is needed for the synthesis of viral genomes and therefore viral particle titer is expected to decrease as polymerase is decreased.
- 30 HBV SEQ ID NO:9 maps to the X gene. Due to the terminal redundancy of all the HBV mRNAs, these effector RNAs have the potential to target all of the HBV viral mRNAs. X protein has many ascribed (non proven) functions. Evidence is emerging, however, that X-gene expression is associated with hepatocellular carcinogenesis, in part related to promotion of

detachment and migration of cells out of the primary tumor site. Since the X gene is often found in integrated HBV sequences in individuals with and without active hepatitis, down-regulation of X gene expression is predicted to ameliorate disease, including the incidence of hepatocellular carcinoma.

5 In general, the more sequences or sequitopes from the different identified sequences that are used (e.g., from SEQ ID NO:1, SEQ ID NO:2, and/or SEQ ID NO:3, plus sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10), the less likely a virus will be able to generate viable escape mutants. Also, the more different mRNAs that can be targeted, the more significant will be the drops in viral titer
10 and disease amelioration.

Desirable combinations for multiepitope or multisequitope dsRNA expression constructs or dsRNA effector molecules, an admixture of dsRNA expression constructs or dsRNA effector molecules , or the concomitant administration of different dsRNA expression constructs or dsRNA effector molecules include the following: Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:5; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from

SEQ ID NO:5 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:9;
Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID
5 NO:5 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from
10 SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:9;
Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus
15 sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9 and SEQ ID NO:10; Sequences from SEQ ID NO:4 and SEQ ID NO:5; Sequences from SEQ ID NO:4 and SEQ ID NO:6; Sequences from SEQ ID NO:4 and SEQ ID NO:7; Sequences from SEQ ID NO:4 and SEQ ID NO:8; Sequences from SEQ ID NO:4 and SEQ ID NO:9; Sequences from SEQ ID NO:4 and SEQ ID NO:10;
20 Sequences from SEQ ID NO:5 and SEQ ID NO:6; Sequences from SEQ ID NO:5 and SEQ ID NO:7; Sequences from SEQ ID NO:5 and SEQ ID NO:8; Sequences from SEQ ID NO:5 and SEQ ID NO:9; Sequences from SEQ ID NO:5 and SEQ ID NO:10; Sequences from SEQ ID NO:6 and SEQ ID NO:7; Sequences from SEQ ID NO:6 and SEQ ID NO:8;
25 Sequences from SEQ ID NO:6 and SEQ ID NO:9; Sequences from SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:7 and SEQ ID NO:9; Sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences from SEQ ID NO:9 and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; and
30 SEQ ID NO:6; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID

SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:9;

Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID

5 NO:4, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:7; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6; SEQ ID NO:8; and SEQ ID NO:9;

10 Sequences from SEQ ID NO:5, SEQ ID NO:6; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:7; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID

15 NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and
SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID
NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10;
Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8;

20 Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8,

25 and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7.

30 SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID
NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID

NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ 5 ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ 10 ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; and Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, 15 SEQ ID NO:9, and SEQ ID NO:10. Preferred in some aspects are sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8, including combinations of sequitopes from SEQ ID NO:5, plus SEQ ID NO:6, plus SEQ ID NO:7, plus SEQ ID NO:8.

In another embodiment, combinations of sequitopes at least 19 contiguous base pairs in length and longer sequences from within any of the aforementioned sequences (e.g., SEQ ID 20 NO:1 through SEQ ID NO:12) may be utilized either in a single dsRNA expression construct or dsRNA effector molecule, an admixture of dsRNA expression constructs or dsRNA effector molecules or through concomitant administration of such dsRNA expression constructs or dsRNA effector molecules to a patient. By a sequence of “at least 19 contiguous base pairs in length” is meant that a sequence or sequitope of at 19 contiguous bases in length is present in 25 double-stranded conformation, or within a double-stranded RNA effector molecule.

As discussed elsewhere herein, a particularly preferred embodiment of the invention utilizes dsRNA expression constructs or vectors to achieve endogenous delivery of the dsRNAs of the invention, especially the multiple different sequences described above. These dsRNAs may be provided e.g., on the same cistron of an expression construct such as a plasmid, on 30 different cistrons of an expression construct, or on different expression constructs or plasmids, e.g., one or more plasmids and/or one or more vectors, including viral vectors. The combination

of different dsRNA effector molecules such as shRNA effector molecules may be provided to a mammalian cell by in vivo expression from a single expression construct such as a plasmid, with each dsRNA effector molecule transcribed from a different expression cassette driven by a different promoter, e.g., an RNA polymerase I promoter and/or an RNA polymerase III
5 promoter, e.g., a type 3 RNA polymerase III promoter such as U6, H1, 7SK, or MRP. In some embodiments, each such different expression cassette may contain a different RNA polymerase III promoter, which may be the same or different, and an RNA polymerase III termination sequence. In another embodiment, a combination of different dsRNA effector molecules such as shRNA effector molecules may be provided to a mammalian cell by in vivo expression from a
10 single expression construct such as a plasmid or a viral vector which comprise an expression cassette comprising multiple different promoters, e.g., an RNA polymerase I promoter and/or an RNA polymerase III promoter, e.g., a type 3 RNA polymerase III promoter such as U6, H1, 7SK, or MRP, and wherein each of such promoters transcribes a different dsRNA effector molecule. Such multiple different dsRNA effector sequences may also be provided to an in vivo
15 mammalian cell exogenously, in any different mixture of one or more dsRNA structures, duplexes and/or harpins, and/or in combination with one or more endogenously expressed dsRNA structures.

Desirable methods of administration of nucleic acids The DNA and/or RNA constructs, e.g., dsRNA effector molecules, of the invention may be administered to the host
20 cell/tissue/organism as “naked” DNA, RNA, or DNA/RNA, formulated in a pharmaceutical vehicle without any transfection promoting agent. More efficient delivery may be achieved as known to those of skill in the art of DNA and RNA delivery, using e.g., such polynucleotide transfection facilitating agents known to those of skill in the art of RNA and/or DNA delivery. The following are exemplary agents: cationic amphiphiles including local anesthetics such as
25 bupivacaine, cationic lipids, liposomes or lipidic particles, polycations such as polylysine, branched, three-dimensional polycations such as dendrimers, carbohydrates, detergents, or surfactants, including benzylammonium surfactants such as benzalkonium chloride. Non-exclusive examples of such facilitating agents or co-agents useful in this invention are described in U.S. Patent numbers 5,593,972; 5,703,055; 5,739,118; 5,837,533; 5,962,482; 6,127,170;
30 6,379,965; and 6,482,804; and International Patent Application No. PCT/US98/22841; the teaching of which is hereby incorporated by reference. U.S. Patents numbers 5,824,538;

5,643,771; and 5,877,159 (incorporated herein by reference) teach delivery of a composition other than a polynucleotide composition, e.g., a transfected donor cell or a bacterium containing the dsRNA-encoding compositions of the invention.

In some embodiments, the dsRNA or dsRNA expression vector is complexed with one or
5 more cationic lipids or cationic amphiphiles, such as the compositions disclosed in US 4,897,355 (Eppstein *et al.*, filed October 29, 1987), US 5,264,618 (Feigner *et al.*, filed April 16, 1991) or US 5,459,127 (Feigner *et al.*, filed September 16, 1993). In other embodiments, the dsRNA or dsRNA expression vector is complexed with a liposome/liposomal composition that includes a cationic lipid and optionally includes another component such as a neutral lipid (see, for
10 example, US 5,279,833 (Rose), US 5,283,185 (Epand), and US 5,932,241).

Particularly desirable methods and compositions for delivery of the oligonucleotide compositions of the invention for pharmaceutical applications, including for targeted delivery to hepatocytes, are described in PCT/US03/14288, filed May 6, 2003, the teaching of which is incorporated herein by reference.

15 Transformation/transfection of the cell for research and other non-therapeutic purposes may occur through a variety of means including, but not limited to, lipofection, DEAE-dextran-mediated transfection, microinjection, calcium phosphate precipitation, viral or retroviral delivery, electroporation, or biolistic transformation. The RNA or RNA expression vector (DNA) may be naked RNA or DNA or local anesthetic complexed RNA or DNA (See U.S.
20 Patent Nos. 6,217,900 and 6,383,512, "Vesicular Complexes and Methods of Making and Using the Same, Pachuk *et al.*, *supra*).

Another desirable delivery technology for the dsRNAs or dsRNA expression constructs of the invention for pharmaceutical applications is the self-assembling Cyclosert™ two-component nucleic acid delivery system, based on cyclodextrin-containing polycations, which
25 are available from Insert Therapeutics, Pasadena, CA. (See Bioconjug Chem 2003 May-Jun; 14 (3): 672-8; Popielarski et al.; "Structural effects of carbohydrate-containing polycations on gene delivery. 3. Cyclodextrin type and functionalization"; as well as Bioconjug Chem 2003 Jan-Feb; 14 (1):247-54 and 255-61.) The first component is a linear, cyclodextrin-containing polycationic polymer, that when mixed with DNA, binds to the phosphate "backbone" of the
30 nucleic acid, condensing the DNA and self assembling into uniform, colloidal nanoparticles that protect the DNA from nuclease degradation in serum. A second component is a surface

modifying agent with a terminal adamantane-PEG molecule, that when combined with the cyclodextrin polymer forms an inclusion complex with surface cyclodextrins and prevents aggregation, enhances stability and enables systemic administration. In addition, targeting ligands to cell surface receptors may be attached to the modifier providing for targeted delivery
5 of DNA directly to target cells of interest. Since hepatocytes are susceptible to HBV and HCV infection, utilizing this method to target delivery of the dsRNA expression constructs of the invention to liver cells is considered especially advantageous. E.g., the asialoglycoprotein receptor (ASGP-R) on mammalian hepatocytes may be targeted by use of synthetic ligands with galactosylated or lactosylated residues, such as galactosylated polymers.

10 In general, targeting for selective delivery of the dsRNA constructs of the invention to hepatocytes is preferred. Targeting to hepatocytes may be achieved by coupling to ligands for hepatocyte-specific receptors. For example, asialo-orosomucoid, (poly)L-lysine-asialo-orosomucoid, or any other ligands of the hepatic asialoglycoprotein receptor (Spiess, Biochemistry 29(43):10009-10018, 1990; Wu et al., J. Biol. Chem. 267(18):12436-12439, 1992;
15 Wu et al., Biotherapy 3:87-95, 1991). Similarly, the oligonucleotides may be targeted to hepatocytes by being conjugated to monoclonal antibodies that specifically bind to hepatocyte-specific receptors. Oligonucleotides may also be targeted to hepatocytes using specific vectors, as described below.

Particularly preferred compositions for delivery of dsRNAs or dsRNA expression
20 constructs of the invention are the multifunctional compositions as described in PCT/US03/14288, filed May 6, 2003, which may include trilactosyl spermine as a ligand for targeting to the ASG Receptor of hepatocytes. Trilactosyl cholesteryl spermine co-complexes with the oligonucleotides of the invention may be prepared and used as described to transfect hepatocytes *in vivo*.

25 The dsRNA oligonucleotides of the invention may be provided exogenously to a target hepatocyte, e.g., prepared outside the cell and delivered into a mammalian hepatocyte. Alternatively, a dsRNA may be produced within the target cell by transcription of a nucleic acid molecule comprising a promoter sequence operably linked to a sequence encoding the dsRNA. In this method, the nucleic acid molecule is contained within a non-replicating linear or circular
30 DNA or RNA molecule, or is contained within an autonomously replicating plasmid or viral vector, or is integrated into the host genome. Any vector that can transfet a hepatocyte may be

used in the methods of the invention. Preferred vectors are viral vectors, including those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses (see, e.g., W089/07136; Rosenberg et al., N. Eng. J. Med. 323(9):570-578, 1990), adenovirus (see, e.g., Morsey et al., J. Cell. Biochem., Supp. 17E, 1993; Graham et al., in Murray, ed., Methods in Molecular Biology: Gene Transfer and Expression Protocols. Vol. 7, Clifton, N.J.: the Human Press 1991: 109-128), adeno-associated virus (Kotin et al., Proc. Natl. Acad. Sci. USA 87:2211-2215, 1990), replication defective herpes simplex viruses (HSV; Lu et al., Abstract, page 66, Abstracts of the Meeting on Gene Therapy, Sep. 22-26, 1992, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), and any modified versions of these vectors. Methods for constructing expression vectors are well known in the art (see, e.g., Molecular Cloning: A Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, N.Y., 1989).

Appropriate regulatory sequences can be inserted into the vectors of the invention using methods known to those skilled in the art, for example, by homologous recombination (Graham et al., J. Gen. Virol. 36:59-72, 1977), or other appropriate methods (Molecular Cloning: A Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, N.Y., 1989).

Upon assembly of a recombinant DNA plasmid dsRNA expression vector on the invention, bacteria are used as “factories” to produce large quantities of the final vector. The E.coli bacterium is frequently used for plasmid fermentation, and it may be advantageous to employ for this purpose E. coli strains having a reduced genome as described in, e.g., Blattner et al., Published U.S. Patent Application No. 2005/0032225, the teaching of which is incorporated herein by reference. The vector manufactured in this manner, isolated and purified according to methods known in the art, can be introduced into living cells with a variety of methods, collectively known as “transfection”, including the methods and compositions described above. Once inside the cell, the promoter elements are recognized by the cellular machinery available for gene transcription and the RNA effector molecules, e.g., shRNAs, will be produced.

Other bacterial strains that may be advantageous for propagating a plasmid expression vector of the invention include the E. coli GT116 Competent Cells available commercially from InvivoGen, San Diego, CA. GT116 is a sbcCD deletion strain specifically engineered to support the growth of plasmid DNAs carrying hairpin structures, such as the plasmids of the invention

engineered to express one or more dsRNA effector molecules which are hairpin RNAs. Hairpin structures are known to be unstable in *E. coli* due to their elimination by a protein complex called SbcCD that recognizes and cleaves hairpins (Connelly et al., Proc. Natl. Acad. Sci. USA 95:7969-74 (1998)). The *sbcCD* and *sbcD* genes are deleted in *E. coli* GT116, which improves
5 its utility for cloning plasmids with hairpin or other palindrome-containing structures.

Promoters Promoters are inserted into the vectors so that they are operably linked, typically but not invariably, 5' to the nucleic acid sequence encoding the dsRNA oligonucleotide. Any promoter that is capable of directing initiation of transcription in a eukaryotic cell may be used in the invention. For example, non-tissue-specific promoters, such
10 as the cytomegalovirus (DeBernardi et al., Proc. Natl. Acad. Sci. USA 88:9257-9261, 1991, and references therein), mouse metallothioneine I gene (Hammer, et al., J. Mol. Appl. Gen. 1:273-288, 1982), HSV thymidine kinase (McKnight, Cell 31:355-365 1982), and SV40 early (Benoist et al., Nature 290:304-310, 1981) promoters may be used. Non-tissue-specific promoters may be used in the invention, as expression of HBV and/or HCV dsRNA in non-liver cells directed by
15 the non-tissue-specific promoters should be harmless to the non-liver cells, because of the specificity of the HBV and HCV dsRNAs of the invention for viral sequences. However, preferred promoters for use in the invention are hepatocyte-specific promoters, the use of which ensures that the RNAs are expressed primarily in hepatocytes. Preferred hepatocyte-specific promoters include, but are not limited to the albumin, alpha-fetoprotein, alpha-1-antitrypsin,
20 retinol-binding protein, and asialoglycoprotein receptor promoters. Viral promoters and enhancers, such as those from cytomegalovirus, herpes simplex viruses (types I and II), hepatitis viruses (A, B, and C), and Rous sarcoma virus (RSV; Fang et al., Hepatology 10:781-787, 1989), may also be used in the invention.

dsRNA expression vectors may include promoters for RNA polymerase I, RNA
25 polymerase II including but not limited to HCMV, SCMV, MCMV, RSV, EF2a, TK and other HSV promoters such as ICP6, ICP4 and ICP0 promoters, HBV pregenomic promoter, RNA pol III promoter, especially type 3 RNA polymerase III promoters, including but not limited to 7SK, U6, and H1, and tRNA promoters, as well as mitochondrial light and heavy strand promoters. Desirably, the dsRNA expression vector comprises at least one RNA polymerase II promoter, for
30 example, a human CMV-immediate early promoter (HCMV-IE) or a simian CMV (SCMV) promoter, at least one RNA polymerase I promoter, or at least one RNA polymerase III

promoter. The promoter may also be a T7 promoter, in which case, the cell further comprises T7 RNA polymerase. Alternatively, the promoter may be an SP6 promoter, in which case, the cell further comprises SP6 RNA polymerase. The promoter may also be one convergent T7 promoter and one convergent SP6 RNA promoter. A cell may be made to contain T7 or SP6

5 polymerase by transforming the cell with a T7 polymerase or an SP6 polymerase expression plasmid, respectively. In some embodiments, a T7 promoter or a RNA polymerase III promoter is operably linked to a nucleic acid that encodes a short dsRNA (e.g., a dsRNA that is less than 200, 150, 100, 75, 50, or 25 basepairs in length). In other embodiments, the promoter is a mitochondrial promoter that allows cytoplasmic transcription of the nucleic acid in the vector

10 (see, for example, the mitochondria) promoters described in WO 00/63364, filed April 19, 2000, and in WO/US2002/00543, filed 9-Jan-2001). Alternatively, the promoter is an inducible promoter, such as a lac (Cronin et al. Genes & Development 15: 1506-1517, 2001), ara (Khlebnikov et al., J Bacteriol. 2000 Dec;182(24):7029-34), ecdysone (Rheogene website), RU48 (mifepristone) (corticosteroid antagonist) (Wang XJ, Liefer KM, Tsai S, O'Malley BW,

15 Roop DR, Proc Natl Acad Sci U S A. 1999 Jul 20;96(15):8483-8), or tet promoter (Rendal et al., Hum Gene Ther. 2002;13(2):335-42 and Larnartina et al., Hum Gene Ther. 2002;13(2):199-210) or a promoter disclosed in WO 00/63364, filed April 19, 2000. Also useful in the methods and compositions of the invention are the structural and chimeric promoters taught in USSN 60/464,434, filed 22-Apr-2003. See also the promoter systems taught in Pachuk, C., and

20 Satishchandran, C., "Multiple-Compartment Eukaryotic Expression Systems," U.S. Provisional Application No. 60/497,304, filed 22-Aug-2003, which are considered particularly desirable in the methods and compositions of the invention.

Liver specific promoters useful in dsRNA expression constructs of the invention include the albumin promoter, the alpha-fetoprotein promoter (especially in liver cancer cells), the alpha-25 1-antitrypsin promoter, hepatitis B promoters, e.g., hepatitis B promoters including promoters for the antigen genes, including core, e antigen, polymerase and X protein.

T7 Promoter/T7 Polymerase Expression Systems A desirable method of the invention utilizes a T7 dsRNA expression system to achieve cytoplasmic expression of dsRNA, (e.g., long or short dsRNA molecules) in vertebrate cells (e.g., mammalian cells). The T7 expression system utilizes the T7 promoter to express the desired dsRNA. Transcription is driven by the T7 RNA polymerase, which can be provided on a second plasmid or on the same plasmid.

Bacteriophage T7 RNA polymerase (T7 Pol) is the product of T7 gene 1, which can recognize its responsive promoter sequence specifically and exhibit a high transcriptase activity. The complete sequence of the T7 genome, with detailed information about the different regions of the bacteriophage, including promoter sequences, is disclosed in Dunn & Studier, 1983, J. Mol. Biol.

5 166(4), 477-535 (see also NCBI 'Genome' database, Accession No. NC 001604). The T7 promoter cannot be utilised by any other RNA polymerase than the polymerase of bacteriophage T7, which shows a stringent specificity for the promoter (Chamberlin et al., 1970, Nature 228:227-231). When utilizing the T7 expression system for expressing dsRNAs, for example, a first plasmid construct that expresses both a sense and antisense strand under the control of

10 10 converging T7 promoters and a second plasmid construct that expresses the T7 RNA polymerase under the control of an RSV promoter can be used. Both the dsRNA and the T7 RNA polymerase could advantageously be expressed from a single bicistronic plasmid construct, particularly when the dsRNA is formed from a single RNA strand with inverted repeats or regions of self-complementarity that enable the strand to assume a stem-loop or hairpin structure

15 15 with an at least partially double stranded region. Individual sense and antisense strands which self assemble to form a dsRNA can be synthesized by a single plasmid construct using, e.g., converging promoters such as bacteriophage T7 promoters placed respectively at the 5' and 3' ends of the complementary strands of a selected sequence to be transcribed. See also, e.g., the teaching of WO 0063364, with respect to T7 dsRNA expression systems, as well as USSN

20 20 60/399,998P, filed 31-Jul-2002 and USSN 60/419,532, filed 18-Oct-2002.

Therapeutic Compositions of the Invention The dsRNAs of the invention, and the recombinant vectors containing nucleic acid sequences encoding them, may be used in therapeutic compositions for preventing or treating HCV and/or HBV infection. The therapeutic compositions of the invention may be used alone or in admixture, or in chemical combination,

25 25 with one or more materials, including other antiviral agents. Currently, lamivudine, adefovir dipivoxil, and interferon alpha have been approved for treatment of HBV, and it is anticipated that the compositions of the invention may be used in combination with these and other drugs that are active against HBV, including emtricitabine (FTC) and entecavir. Since dsRNAs against HBV and/or HCV act through a novel mechanism (dsRNA-mediated gene silencing/RNAi),

30 30 combination therapy of the agents of the invention and other antivirals is expected to significantly increase the efficacy of therapy while substantially reducing the development of

drug resistance, e.g., the development of lamivudine resistance, a problem of major concern with long term lamivudine therapy. Currently, interferon and ribavirin are licensed for treatment of HCV, and as for HBV, it is anticipated that the compositions of the invention may be used in combination with these and other drugs that are active against HCV. Specific dosage regimens 5 involving therapy with such multiple agents can be determined through routine experimentation by those of ordinary skill in the art of clinical medicine.

Formulations will desirably include materials that increase the biological stability of the oligonucleotides or the recombinant vectors, or materials that increase the ability of the therapeutic compositions to penetrate hepatocytes selectively. The therapeutic compositions of 10 the invention may be administered in pharmaceutically acceptable carriers (e.g., physiological saline), which are selected on the basis of the mode and route of administration, and standard pharmaceutical practice. One having ordinary skill in the art can readily formulate a pharmaceutical composition that comprises an oligonucleotide or a gene construct. In some cases, an isotonic formulation is used. Generally, additives for isotonicity can include sodium 15 chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vasoconstriction agent is added to the formulation. The pharmaceutical preparations according to the present invention are provided sterile and pyrogen free. Suitable pharmaceutical carriers, as well as pharmaceutical necessities for use in pharmaceutical 20 formulations, are described in Remington: The Science and Practice of Pharmacy (formerly Remington's Pharmaceutical Sciences), Mack Publishing Co., a standard reference text in this field, and in the USP/NF.

Routes of administration include, but are not limited to, intramuscular, intraperitoneal, 25 intradermal, subcutaneous, intravenous, intraarterially, intraocularly and oral as well as transdermally or by inhalation or suppository. Preferred routes of administration include intravenous, intramuscular, oral, intraperitoneal, intradermal, intraarterial and subcutaneous injection.

Targeted transfection of hepatocytes *in vivo* for delivery of dsRNAs against HBV and/or HCV may be accomplished through IV injection with a composition comprising a DNA or RNA 30 expression vector as described herein complexed with a mixture (e.g., a 35%/65% ratio) of a lactosyl spermine (mono or trilactosylated) and cholesteryl spermine (containing spermine to

DNA at a charge ratio of 0.8). Such compositions are especially useful for pharmaceutical applications and may readily be formulated in a suitable sterile, non-pyrogenic vehicle, e.g., buffered saline for injection, for parenteral administration, e.g., IV (including IV infusion), IM, SC, and for intraperitoneal administration, as well as for aerosolized formulations for pulmonary delivery via inhalation. In certain formulations, a DNA expression construct of the invention may be complexed with an endosomolytic spermine such cholesteryl spermine alone, without a targeting spermine; some routes of administration, such as intraperitoneal injection or infusion, may achieve effective hepatic delivery and transfection of a DNA construct of the invention, and expression of RNA effector molecules, e.g., multiple dsRNA hairpins effective against HBV and/or HCV.

Intraperitoneal administration (e.g., ultrasound guided intraperitoneal injection) of a sterile pharmaceutical composition comprising dsRNA effector molecules and/or dsRNA expression constructs which provide dsRNA effector molecules against HBV and/or HCV in a specially formulated delivery vehicle may be an advantageous route of delivery to promote uptake by liver cells, including hepatocytes. In some compositions the dsRNA expression construct may be complexed with an appropriate transfection enhancing agent, e.g., with a mixture of a lactosyl spermine (mono or trilactosylated) and cholesteryl spermine, or in other compositions with an endosomolytic spermine such cholesteryl spermine alone, without a targeting spermine. The volume, concentration, and formulation of the pharmaceutical composition as well as the dosage regimen may be tailored specifically to maximize cellular delivery while minimizing toxicity such as an inflammatory response. E.g, relatively large volumes (5, 10, 20, 50 ml or more) with corresponding low concentrations of active ingredients, as well as the inclusion of an anti-inflammatory compound such as a corticosteroid, may be utilized if desired. Formulations as known to those of skill in the art of pharmaceutics may also be utilized to provide sustained release of dsRNA effector molecules and/or dsRNA expression constructs of the invention.

dsRNAs or dsRNA expression constructs may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or “microprojectile bombardment gene guns”. Intraperitoneal injection may be accomplished, e.g., with a traditional syringe, with placement of the needle advantageously guided by ultrasound or a similar technique. Alternatively, the dsRNA and/or dsRNA expression construct may be introduced by

various means into cells that are removed from the individual. Such means include, for example, ex vivo transfection, electroporation, microinjection and microprojectile bombardment. After the gene construct is taken up by the cells, they are reimplanted into the individual. It is contemplated that otherwise non-immunogenic cells that have gene constructs incorporated 5 therein can be implanted into the individual even if the host cells were originally taken from another individual.

In HBV infected individuals it is anticipated that the dsRNA compositions of the invention may be useful as a pre-treatment in conjunction with therapeutic vaccination protocols designed to boost immunity against the virus. It is also anticipated that the dsRNA compositions 10 of the invention may be useful for prophylaxis in a regimen of periodic administrations to individuals who because of occupational or other potential for exposure are considered at high risk of exposure to HBV and/or HCV, e.g., fire, emergency, and health care personnel. Such an effective prophylactic regime may include administration of a composition that provides an HBV and/or HCV dsRNA of the invention, e.g., weekly, biweekly, monthly, bimonthly, every three 15 months, every four months, semi-yearly, or yearly, as can be determined through routine experimentation by those of skill in the art of clinical medicine. The ability of a dsRNA expression vector such as a plasmid or viral vector to express the dsRNAs of the invention over a relatively prolonged period of time, expected to be in the range of weeks to months, is considered to be advantageous for this and other applications.

20 Dosage of dsRNAs For administration of dsRNA (e.g., a short dsRNA to inhibit toxicity or a short or long dsRNA to silence a gene) to an animal, typically between 10 mg to 100 mg, 1 mg to 10 mg, 500 µg to 1 mg, or 5 µg to 500 µg dsRNA is administered to a 90-150 pound person/animal (in order of increasing preference). For administration of a vector encoding 25 dsRNA (e.g., a short dsRNA to inhibit toxicity or a short or long dsRNA to silence a gene) to an animal, typically between 100 mg to 300 mg, 10 mg to 100 mg, 1 mg to 10 mg, 500 µg to 1 mg, or 50 µg to 500 µg dsRNA expression vector or construct is administered to a 90-150 pound person/animal (in order of increasing preference). The dose may be adjusted based on the weight 30 of the animal. In some embodiments, about 1 to 10 mg/kg or about 2 to 2.5 mg/kg is administered. Other doses may also be used, as determined through routine experimentation by those of skill in the art of clinical medicine.

For administration in an intact animal, e.g., a human subject infected with HBV and/or HCV, between 1 mg and 100 mg, typically between 1 mg and 10 mg, between 10 ng and 50 µg, between 50 ng and 100 ng, or between 100 ng and 5 µg of dsRNA or DNA encoding one or more dsRNA effector molecules is used. In desirable embodiments, approximately 10 µg of a

5 DNA or 5 µg of dsRNA is administered to the animal. In a desirable embodiment, a pharmaceutical composition for parenteral administration is prepared containing 10 mg of a plasmid dsRNA expression construct of the invention (in some formulations complexed with an appropriate transfection facilitating agent such as cholesteryl spermine or a mixture of cholesteryl spermine/trilactosyl spermine) in 25 ml of a suitable sterile vehicle for injection such

10 as Normal Saline Injection, D5W, D5%/0.45% NaCl, D5%/0.2% NaCl, etc., and injected intraperitoneally over 5 to 10 minutes, with needle placement guided by ultrasound or a similar technology. Administration may be repeated periodically, e.g., weekly or monthly, as required. With respect to the methods of the invention, it is not intended that the administration of dsRNA or DNA encoding dsRNA to cells or animals be limited to a particular mode of administration,

15 dosage, or frequency of dosing; the present invention contemplates all modes of administration sufficient to provide a dose adequate to inhibit gene expression, prevent a disease, or treat a disease.

If desired, short dsRNA is delivered before, during, or after the exogenous delivery of dsRNA (e.g., a longer dsRNA) that might otherwise be expected to induce cytotoxicity. See the
20 teaching of USSN 10/425,006, filed 28-Apr-2003, "Methods of Silencing Genes Without Inducing Toxicity", Pachuk.

Therapeutic Advantages of the Invention as it Relates to Worldwide Disease Incidence and Viral Variability

25 The mutability of the hepatitis C virus genome, and to a lesser but significant extent, the hepatitis B virus genome, has been described above as presenting challenges to the design of nucleic acid based therapeutics against these viral agents. The inventors have painstakingly aligned thousands of individual HCV and HBV sequences, originally deriving from thousands of human viral isolates from widely divergent geographic areas worldwide. In doing so, the instant
30 invention has identified and specified preferred sequences which are utilized singly and in

combination in dsRNA effector molecules which target the least mutable regions of the genome of HCV and/or HBV.

The two-fold rationale for this has been discussed above, primarily in terms of ensuring that during the course of infection of a patient with HBV or HCV, the therapeutic of the invention will remain potent against the virus even as it mutates during the course of disease in a given patient. However, the second part of this rationale for deriving and using highly conserved sequences for design of dsRNA-based therapeutic applications, is that this also increases the certainty that the therapeutic dsRNA effector molecules of the invention, particularly the methods and compositions of the invention which utilize combinations of highly conserved sequences in dsRNA effector molecules against HBV and/or HCV, will be able to treat the viral infection present in individuals from a global variety of different ancestries, genetic makeup, and geographical distribution, which are known to manifest in clusters of viral variants based on such factors. Thus, a key feature of the therapeutic utility and novelty of this invention lies in the method of derivation of the preferred sequences and embodiments, and not simply in the demonstration that any particular HCV or HBV dsRNA sequence can inhibit viral replication of one or a few chosen viral isolates (or their cognate replicons) in a laboratory experiment, i.e., in a cell line or animal model, not necessarily reflective of the broad diversity of the HBV and/or HCV virus worldwide, or even in a particular infected individual over the course of infection.

For example, the hepatitis B virus has four subtypes of surface antigen, namely adw, ayw, adr and ayr. While lamivudine is considered an effective therapy for chronic Hepatitis B, a recent study of HBV resistance demonstrated a 20-fold increase in resistance in the adw subtype, compared to the ayw subtype. B Zollner et al. “20-fold Increase in Risk of Lamivudine [Epivir HBV] Resistance in Hepatitis B Virus Subtype adw”; *The Lancet*. 2001; 357: 934-935. In contrast to such conventional antiviral agents, the dsRNA agents of the invention, (e.g., dsRNA effector molecules and expression constructs of the invention, especially when used in combination as taught herein) which utilize HBV and/or HCV sequences highly conserved across such geographical genetic variants are expected to exhibit highly advantageous antiviral activity.

Similarly, HCV is also known for having a wide range of geographically divergent viral genotypes, subtypes, quasispecies, with the following current general global patterns of genotypes and subtypes:

- 1a - mostly found in North & South America; also common in Australia
1 b - mostly found in Europe and Asia.
2a - is the most common genotype 2 in Japan and China.
2b - is the most common genotype 2 in the US and Northern Europe.
5 2c - the most common genotype 2 in Western and Southern Europe.
3a - highly prevalent here in Australia (40% of cases) and South Asia.
4a - highly prevalent in Egypt
4c - highly prevalent in Central Africa
5a - highly prevalent only in South Africa
10 6a - restricted to Hong Kong, Macau and Vietnam
7a and 7b - common in Thailand
8a, 8b & 9a - prevalent in Vietnam
10a & 11a - found in Indonesia
- Accordingly, the highly conserved sequences of the invention, which are expected to be
15 conserved among most if not all of these divergent HCV genotypes and subtypes worldwide, including 1a, 1b, 2a, 2b, and 2c, are considered highly effective therapeutics agents, e.g., when utilized as dsRNA effector molecules, especially combinations thereof, and dsRNA expression vectors capable of expressing such dsRNAs.

Applicants specifically incorporate the entire content of all cited references in this
20 disclosure. Further, when an amount, concentration, or other value or parameter is given as either a range, preferred range, or a list of upper preferable values and lower preferable values, this is to be understood as specifically disclosing all ranges formed from any pair of any upper range limit or preferred value and any lower range limit or preferred value, regardless of whether ranges are separately disclosed. Where a range of numerical values is recited herein, unless
25 otherwise stated, the range is intended to include the endpoints thereof, and all integers and fractions within the range. It is not intended that the scope of the invention be limited to the specific values recited when defining a range.

EXAMPLES

The following Examples are provided as illustrative only. All references mentioned
30 within this disclosure are specifically incorporated herein by reference in their entirety.

EXAMPLE 1Silencing HBV replication and expression in a replication
competent cell culture model

Brief description of cell culture model: A human liver-derived cell line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession V01460. Following internalization into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg (Hepatitis B Surface Antigen) particles into the media of transfected cells. This transfection model reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression and replication.

Using this model, cells were co-transfected with the infectious molecular clone of HBV and various eiRNA constructs (dsRNA expression constructs). The cells were then monitored for loss of HBV expression and replication as described below. Details on the vector and encoded RNAs used in this experiment are provided at the end of this example.

Experiment 1:

The following is an example of an experiment that was performed using eiRNA vectors (dsRNA expression vectors) encoding sequences derived from GenBank accession number V01460.

HBV sequences in these described eiRNA vectors were highly conserved sequences identified as described elsewhere herein, which also exhibited activity as siRNAs (See, Pachuk, C., "Methods and Constructs for Evaluation of RNAi targets and Effector Molecules," PCT/US2004/005065, filed 25-Feb-2004). The particular eiRNA backbone vector used for this experiment was a proprietary vector containing a U6 promoter to drive expression of the encoded RNAs. Each vector encoded only one short hairpin RNA (shRNA). The shRNA coding sequence was followed by an RNA pol III termination sequence. Sequences of the U6 promoter, RNA pol In

termination signal, and encoded shRNAs are all shown at the end of the example. Similar vectors containing U6 promoters and RNA pol III termination signals are commercially available such as the “siLentGene-2 Cloning Systems” vector from Promega, Inc., Madison, Wis. One of ordinary skill in the art can also create them according to the information provided herein. It is
5 expected that similar results would also be obtained using other expression and promoter systems especially those vectors with RNA pol III promoters that are not U6, for example H1 promoters or 7SK promoters.

Experimental Procedure: Transfection.

10 Huh7 cells cultured in RPMI-1640 media were seeded into six-well plates at a density of 3×10^5 cells/well. All transfections were performed the day after cell seeding using LipofectamineTM (InVitrogen, Carlsbad, Cal.) according to the manufacturer's directions. In this experiment, cells were transfected with 500 ng of the infectious HBV plasmid ayw subtype (“pHBV2”) (GenBank Accession # V01460) and 500 ng, 300ng, 250ng, 120 ng, 100 ng, 50ng, or
15 10 ng of an eiRNA construct. DNA was held constant/transfection at 2.5 μ g by including an inert plasmid DNA, pGL3-Basic (Promega, Madison Wis.) in amounts that brought the total DNA in the transfection to 2.5 μ g. For example, in transfections receiving 500 ng of HBV DNA and 500 ng of an eiRNA construct, 1.5 μ g pGL3 was added to the transfection. Prior to transfection, media was removed from the cells and the cells washed with Opti-MEM®
20 (InVitrogen Life Technologies, Carlsbad, Cal.). 800 μ l of Opti-MEM® was then added to each well of cells followed by the addition of the transfection mix. Seventeen to nineteen hours post-transfection, the transfection mix and Opti-MEM® were removed from cells and replaced with 2 mL culture media/well. At 3, 6, and 10 days after transfection, the media was removed from cells and stored at -70 °C. The media was replaced with 2 mL of fresh culture media on days 3
25 and 6. All transfections were carried out in duplicate. Two sets of control transfections were also performed: HBV DNA alone (500 ng HBV DNA plus 2 μ g pGL3) and HBV DNA with a control eiRNA construct (500 ng HBV DNA, 1 μ g control eiRNA construct, and 1.0 μ g pGL3 DNA).

Monitoring cells for loss of HBV expression.

Following transfection, cells were monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion. Cells were monitored by assaying the media of transfected cells (and a media control) at days 3, 6, and 10 post-transfection. The Auszyme® ELISA, commercially available from Abbott Labs (Abbott Park, III.), was used to detect surface Ag (sAg) according to the manufacturer's instructions. sAg was measured since surface Ag is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected infectious HBV clone and from HBVcccDNA produced during infection *in vivo*. Since surface Ag synthesis can continue with deleterious effects in the absence of HBV replication, it is important to down-regulate not only viral replication but also replication-independent synthesis of sAg.

Results:

Cells transfected with the HBV-specific eiRNA constructs described at the end of this example all induced a decrease in sAg levels relative to the controls. The level inhibition is shown in the accompanying FIG. 2-8 corresponding to data found in Tables 2-8. Note that the sequences identified as 788-808 and 807-827 only lowered surface Ag levels by 30% and 50% respectively at 500 ng doses. These are the only two eiRNAs that do not target the sAg mRNA; instead they target the 3.1 Kb HBV mRNAs and therefore reduce sAg levels indirectly. The 30% to 50% reduction in sAg observed when these other HBV RNAs are targeted is considered a strong indication that these eiRNA constructs are efficacious.

20 HBV-specific eiRNAs used in this experiment

The eiRNA vectors encode the HBV sequences listed in Table 1. The sequences are shown as well as their map coordinates on GenBank accession number V01460. At the rightmost part of the table is the SEQ ID NO that these sequences map within. The sequence of the encoded RNA is 5'GGTCGAC (a sequence that is *per se* unimportant, but is derived from 25 the polylinker sequence of the particular vector used) followed by a first sense or antisense HBV sequence followed by the loop sequence (underlined in Table 1) followed by a second HBV sequence, which is the complement to the first HBV sequence. Note that the loop structure does not need to be a fixed sequence or length, and we have used several loop sequences with no significant impact on the functioning of the eiRNA construct. The second HBV sequence is

followed by a string of T residues, e.g., 1, 2, 3, or more Ts, that function as the termination signal for RNA pol III.

Table 1

HBV-AYW coordinates*	SEQ ID NO		Maps within SEQ ID NO
788-708	14	CGTCTGCGAGGCAGGGAGTTAGAGAACTTAACCCCTGCCCTCGCAGACG	5, 6, 7, or 8
807-827	15	TTCTTCTCTAGGGGACCTGCAGAGAACCTGCAGGTCCCCTAGAAGAAGAA	5, 6, 7, or 8
1291-1311	16	AAGCCACCCAAGGCACAGCTTAGAGAACCTTAAGCTGTGCCTGGTGGCTT	4
1299-1319	17	CAAGGCACAGCTGGAGGCTTAGAGAACCTTAAGCCTCCAAGCTGTGCCTG	4
1737-1757	18	GGATTAGCGCCGACGGACGAGAGAACCTCGTCCCCTGGCGCTGAATCC	10
1907-1927	19	TTCCCGAGTATGGATCGGCAGAGAGAACCTCTGCCATCCATACTGCGGAA	3
1912-1932	20	CAGTATGGATCGGCAGAGGAGAGAGAACCTTCTCTGCCATCCATACTG	3
1943-1963	21	TCCACGCATGCGCTGATGCCAGAGAACCTGGCCATCAGCGCATGCGTGGAA	3
1991-2011	22	TGCGTCAGCAAACACTTGGCAAGAGAACCTTGCAAGTGTGCTGACGCA	3
2791-2811	23	AAAACGCCGCAGACACATCCAAGAGAACCTTGGATGTGCTGCCGTTTT	2
2791-2811mut	24	AAAACACCACACGCATCCAAGAGAACCTTGGATGCGTGTGGTTTT	2
2912-2932	25	TTGAGAGAACGTCCACCACGAGAGAGAACCTCTCGTGGACTCTCTCAA	1
2919-2939	26	AAGTCCACCACGAGTCTAGACAGAGAACCTGTCTAGACTCGTGGACTT	1
799-779	49	GCCTCGCAGACGAAGGTCTCAAGAGAACTTGAGACCTCGTCTCGAGGC	

* nucleotide coordinates refer to Genbank accession number V01460.

A diagram of the transcribed RNA structure is shown in **FIG. 9**.

SEQ ID NO:13 is the nucleotide sequence of U6 promoter. Nucleotide sequence of RNA pol III terminator: 5' – TTTTT – 3'.

- The HBV sequitopes of Table 1 (without their respective complement sequence and
- 5 without the “loop” or linker sequence utilized in a “hairpin” or stem-loop dsRNA effector molecule) are shown in Table 1A below. Each such HBV sequitope, together with its complementary sequence, optionally with an appropriate loop or linker sequence as taught herein, may be utilized in a dsRNA effector molecule of the invention (e.g., a duplex dsRNA or hairpin dsRNA effector molecule, or encoded within a dsRNA expression vector). In one aspect,
- 10 multiple (e.g., 2, 3, 4, 5, 6, or more) of such dsRNA short hairpin effector molecules are encoded in a single expression vector, e.g., a plasmid expression vector, each under the control of a different promoter, e.g., a polymerase III promoter, as described elsewhere herein.

TABLE 1A

HBV-AYW coordinates <i>Genbank accession number V01460*</i>	SEQ ID NO	Conserved HBV Sequitope	Maps within SEQ ID NO
788-808	50	CGTCTGCGAGGCGAGGGAGTT	5, 6, 7, or 8
807-827	51	TTCTTCTTCTAGGGGACCTGC	5, 6, 7, or 8
1291-1311	52	AAGCCACCCAAGGCACAGCTT	4
1299-1319	53	CAAGGCACAGCTTGGAGGCTT	4
1737-1757	54	GGATTTCAGCGCCGACGGGACG	10
1907-1927	55	TTCCGCAGTATGGATCGGCAG	3
1912-1932	56	CAGTATGGATCGGCAGAGGAG	3
1943-1963	57	TCCACGCATGCGCTGATGGCC	3
1991-2011	58	TGCGTCAGCAAACACTTGGCA	3
2791-2811	59	AAAACGCCGCAGACACATCCA	2
2912-2932	60	TTGAGAGAAAGTCCACCACGAG	1
2919-2939	61	AAGTCCACCACGAGTCTAGAC	1
799-779	62	GCCTCGCAGACGAAGGTCTCA	

15

Tables and Graphs.

HBsAg was measured as described above and plotted in **FIG. 2-8** corresponding to the data in Tables 2-8. The amount of eiRNA construct is shown in parentheses following the name

of the eiRNA construct and is in μg amounts. For example, 2791(0.5) means that 0.5 μg or 500 ng of eiRNA construct 2791-2811 (see Table 1) was used in the transfection. The percent inhibition relative to the control is also shown in the tables below and it is specific for the day 10 measurement. Note that the 4th set of data in this example in which 1299 was evaluated at 500 ng has only two timepoints, days 3 and 6, because the evaluation was not carried out at day 10. The percent inhibition for this experiment was shown for day 6 data. Data is shown as raw OD 5 data collected as described by the manufacturer of the Auszyme ELISA assay kit used to measure sAg. Not shown are the 50 ng data for 2791-2811 and the 10ng data for 1907-1927. Each of these doses inhibited HBsAg expression by about 50% relative to the control.

10 Table 2

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.339	1.88	3.268	-----
2791(0.5)	0.101	0.263	0.333	89.8

Table 3

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	1.169	4.445	10.18	-----
2791(0.5)	0.442	0.743	1.3	87.2
2791Mut(0.5)	1.136	4.305	10.595	-----

15

Table 4

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.375	1.952	4.005	-----
2791mut(1)	0.421	1.847	4.753	-----
HCV(1)	0.445	1.805	3.933	-----
788(0.5)	0.255	1.195	2.778	30.6
807(0.5)	0.254	1.326	2.015	49.7
1907(0.25)	0.052	0.113	0.365	90.9
1912(0.25)	0.138	0.208	0.517	87.1
1943(0.25)	0.099	0.233	0.506	87.4
1991(0.25)	0.075	0.152	0.291	92.7
2912(0.25)	0.095	0.183	0.331	91.7

Table 5

5

	Day 3	Day 6	% Inhibition relative to control
pHBV2	0.474	1.513	-----
1299(0.5)	0.439	0.699	53.8

Table 6

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.33	1.617	2.88	-----
2791(0.3)	0.103	0.192	0.349	87.9
1737(0.3)	0.051	0.094	0.232	91.9
1291(0.12)	0.239	0.587	1.195	58.5
1907(0.12)	0.043	0.086	0.356	87.6
2919(0.12)	0.218	0.565	1.09	62.2

Table 7

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.741	2.53	5.383	-----
2791(0.3)	0.223	0.256	0.458	91.5
1737(0.1)	0.212	0.351	0.549	89.8
1907(0.1)	0.067	0.149	0.468	91.3
1991(0.1)	0.067	0.16	0.345	93.6

Table 8

5

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.864	4.414	8.344	-----
1907(0.05)	0.17	0.538	1.396	83.3
2919(0.1)	0.368	1.044	1.908	77.1
1291(0.2)	0.573	1.654	1.896	77.3

Experiment 2:

Background: The same cell culture model was used to evaluate the additive effects of adding two eiRNA constructs. In this experiment 2791-2811 and 2919-2939 were evaluated.

- 10 They were evaluated separately at two doses: 10 ng and 25 ng, and in combination at 10 ng (5 ng of 2791-2811 plus 5 ng of 2919-2939) and at 25 ng (12.5 ng 2791-2811 plus 12.5ng 2919-2939). An additive effect is observed, for example, when half the inhibition seen with 25 ng 2791-2811 plus half the inhibition seen with 25 ng 2919-2939 is about equal to the inhibition seen of the 25 ng combination dose. This is important because while one may not be gaining inhibition over 15 the use of a single eiRNA construct at the 25 ng dose, the use of two or more eiRNA sequences is very important in preventing the generation of viral escape mutants.

Experimental Procedure: Transfection.

Huh7 cells were seeded into six-well plates at a density of 3×10^5 cells/well. All transfections were performed the day after cell seeding using Lipofectamine™ (InVitrogen) according to the manufacturer's directions. In this experiment, cells were transfected with 500 ng of the infectious HBV plasmid ayw subtype (GenBank Accession # V01460) and 25 ng or 10 ng of two separate eiRNA constructs or a combination of these two eiRNA constructs at a total

of 25 ng or 10 ng. DNA was held constant/transfection at 2.5 µg by including an inert plasmid DNA, pGL3, in amounts that brought the total DNA in the transfection to 2.5 µg. For example, in transfections receiving 500 ng of HBV DNA and 10 ng of an eiRNA construct, then 1.99 µg pLUC was added to the transfection. Prior to transfection, media was removed from the cells
 5 and the cells washed with Opti-MEM® (InVitrogen Life Technologies). 800 µl of Opti-MEM® was then added to each well of cells followed by the addition of the transfection mix. Seventeen to nineteen hours post-transfection, the transfection mix and Opti-MEM® was removed from cells and replaced with 2 mL culture media/well. At 4, 8, and 11 days after transfection, the media was removed from cells and stored at -70 °C. The media was replaced with 2 mL of fresh
 10 culture media on days 4 and 8. All transfections were carried out in duplicate. Two sets of control transfections were also performed: HBV DNA alone (500 ng HBV DNA plus 2 µg pGL3), and HBV DNA with a control eiRNA construct (500 ng HBV DNA, 500 ng control eiRNA construct and 1.5µg pGL3. DNA).

Results:

15 Results are shown in Table 9, and the corresponding graph found in **FIG. 10**. Combining 2791-2811 and 2919-2939 showed at least equal effects to administration of 2791-2811 or 2919-2939 alone. It is expected that similar advantages will be seen by combining two or more dsRNAs directed to different HBV sequences from the same and/or different HBV genes.

20 Table 9

	Day 4	Day 8
pHBV ₂	3.74	15.03
2791 @ 25 ng	2.49	9.63
2919 @ 25 ng	2.55	10.07
2791 + 2919 @ 25 ng	2.73	10.91

Experiments 3 and 4

Silencing of HBV in a mouse model.

Summary: Two of the eiRNA vectors described in confirmatory experiment 1 were
 25 assessed for their ability to silence an HBV replicon in a mouse model. These vectors were the 2791-2811 and the 1907-1927 vectors. Both vectors were found to silence HBV in the mouse model to a similar extent as they silenced in the cell culture model. The ability to silence this

HBV replicon in mice by other therapeutics has been demonstrated to be a predictor of human efficacy [4].

Animal Model Background:

Chimpanzees represent the only animal model in which to study human HBV infectivity.

- 5 A mouse model is available, however, in which HBV expression and replication occur. This model has been invaluable for the evaluation of anti-HBV therapeutic agents not only targeted against viral replication but also against RT-independent expression of antigen. In this model, replication competent HBV is expressed transiently from episomal HBV DNA. This model is created by introducing replication competent HBV DNA into mouse liver by hydrodynamic
10 delivery [1].

The aim of the following experiment was to test two of the vectors encoding HBV-specific sequences evaluated in Experiment 1 for efficacy in a mouse model even though there were not expected to be HBV-sequence-related efficiency differences between the cell culture and mouse models. This experiment utilized hydrodynamic delivery as a method to co-deliver
15 replication competent HBVayw plasmid (Example 1, confirmatory experiment 1) with an effector HBV-specific eiRNA expression vector. Hydrodynamic delivery is ideal for these first studies because it results in efficient delivery of nucleic acid to the liver [5].

Experiments.

Hydrodynamic Delivery Studies: Experiment 3.

- 20 All animals were hydrodynamically injected with 7.5 pg infectious HBVayw plasmid (described in confirmatory Example 1). Following internalization into hepatocytes and nuclear localization, transcription of HBVayw plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication [1]. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse
25 transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the sera of injected animals. Experimental animals were co-injected with 10 µg 2791-2811. A second group of control animals were injected with 10 pg of an irrelevant eiRNA construct. All animals were also co-injected with 2.5 µg of a GFP reporter plasmid (Clontech, Palo Alto, Cal.). Expression of GFP mRNA in the livers of injected mice served as a control to normalize
30 results against the mouse model transfection efficiency. Total DNA injected in animals was kept

at a constant 20 µg by including pGL3, an inert filler DNA (Promega, Madison, Wis.). All DNA was formulated and injected according to the methods described in Yang et al. [1]. There were 5 animals per group. The DNAs and amounts of DNA injected per animal are shown in Table 10.

5 Table 10

Group	HBV DNA	GFP DNA	eiRNA	pGL3
1	7.5 µg	2.5 µg	10 µg 2791	0 µg
2	7.5 µg	2.5 µg	10 µg control	0 µg
3	7.5 µg	2.5 µg	0 µg	10 µg

Timepoints of analysis were selected based on published results from Dr. Chisari's laboratory [1], which detail the kinetics of HBVayw plasmid replication in mice following hydrodynamic delivery. Serum was assayed for the presence of HBsAg on days 1, 2, 3, and 4
10 post-injection. Assays were performed as described for the cell culture model of HBV replication. The presence of HBV RNA in liver samples was ascertained by Northern blot analysis on day 2 following injection using procedures developed in Dr. Chisari's laboratory [1] and normalized to endogenous GAPDH RNA levels and GFP mRNA levels using conventional techniques, or a quantitative RT-PCR assay for HBV RNAs containing sAg coding sequences
15 using standard techniques. RT-PCR is more quantitative than Northern Blot analysis and has a larger dynamic window than does Northern Blot analysis.

Downregulation of both HBV RNA by Northern Blot analysis and HBsAg were seen in mice injected with 2791-2811. See **FIG. 11**. Also not shown, quantitative RT-PCR demonstrated the presence of 867 HBV RNA molecules in the livers of control mice and 57
20 molecules of HBV RNA in 2791-2811 treated mice, a 15-fold downregulation.

Table 11

Group	Mouse	3.5 kb	2.1 kb	GFP	HBV total	HBV/GFP	Group Average	Std. Dev
10 µg 2791	1	182360	1440614	4044344	1622974	4.0	3.0	0.76
	2	392294	3161703	9954889	3553997	3.6		
	3	268673	3114347	15317275	3383020	2.2		
	4	394799	3909096	16806285	4303895	2.6		
	5	362182	4439430	18306755	4801612	2.6		
HBV only	21	2412562	8720964	3860082	11133526	28.8	17.4	7.40
	22	2170741	7958388	6110744	10129129	16.6		
	23	2713213	12060855	9633404	14774068	15.3		
	24	1924373	7243024	11042915	9167397	8.3		
	25	1464641	5726217	3968243	7190858	18.1		

Table 12

NUC5_HBsAg		HBsAG (ng/ml)			
		d1	d2	d3	d4
HBV	2	2810	6793	8422	8517
	3	2344	8332	8089	8743
	4	1684	8788	9064	8876
	5	2318	9378	8597	8480
	29	1066	5038	5153	5925
	grp ave=>	2044	7666	7865	8108
Std Dev=>		678	1754	1556	1231
eiHCV	6	2554	8048	9233	8870
	9	2267	8420	9535	8338
	10	1704	8258	8761	7840
	30	1362	4171	5406	4920
	grp ave=>	1972	7224	8234	7492
	Std Dev=>	538	2041	1912	1765
2791	11	1262	2823	2276	2080
	12	1222	2549	2858	1593
	14	1056	1933	1143	792
	15	1275	8320	1920	2068
	27	779	4771	3782	1252
	grp ave=>	1119	4079	2396	1557
Std Dev=>		209	2598	993	551

5

Hydrodynamic Delivery Studies: Experiment 4.

This experiment was similar to the Experiment 3 of Example 1 except that two eiRNA constructs were evaluated: 2791-2811 and 1907-1927. In this experiment, HBsAg was measured on days 1 and 4 using the assay already described herein.

10

Table 13

NUC6_HBsAg		HBsAG (ng/ml)	
		d1	d4
HBV	2	6147 ^	36,953 ^
	3	6234 ^	42,542 ^
	4	4658	33,061 ^
	5	5077 ^	29,389^
	grp ave=>	5529	35486
Std Dev=>		784	5627
eiHCV	6	1901	11,236
	7	6286 ^	29,637 ^
	8	1023	6,345
	grp ave=>	3070	15739
	Std Dev=>	2820	12282
2791	11	3966	5009
	13	4705	7347 ^
	14	2289	4538
	15	2427	4217
	grp ave=>	3347	5278
Std Dev=>		1182	1417
1907	16	4954	7203 ^
	18	2982	6917 ^
	19	3436	7568 ^
	20	2246	5135 ^
	grp ave=>	3405	6706
Std Dev=>		1143	1081

5

A Four-Promoter RNA polymerase III-based expression construct for production of shRNAs which reduce Hepatitis B RNA production and replication.

10 As described in more detail in PCT/US05/29976 filed 23-August-2005, a plasmid expression vector, pHB4, containing 4 polymerase III promoter short hairpin dsRNA expression cassettes was constructed. Each expression cassette included a polymerase III promoter operably linked to a sequence encoding a short hairpin dsRNA, and a polymerase III termination sequence. The polymerase III promoters were U6, 7SK, and two copies of a 7SK sequence
15 variant promoter, designated 7SK-4A. Each short hairpin dsRNA sequence comprised a double-

stranded stem region homologous and complementary to a highly conserved HBV sequence as taught herein. The four dsRNA effector molecules comprised the sequences of SEQ ID NO: 49; SEQ ID NO: 23; SEQ ID NO:19; and SEQ ID NO: 18; which comprise, respectively; the sequences of SEQ ID NO: 62; SEQ ID NO: 59; SEQ ID NO:55; and SEQ ID NO: 54. As described in more detail in Example 1 of PCT/US05/29976, however, the sequence encoding the dsRNA hairpin effector molecule was inserted into an expression cassette of the plasmid expression vector at a restriction site which in effect resulted in several additional nucleotides being added to the 5' end of the ultimate transcript. The predicted transcript which includes the dsRNA hairpin actually contains additional 5' and 3' sequences: a 5' leader consisting of 6 bases (e.g., the Sal I or Hind III or other chosen recognition sequence), followed by the dsRNA hairpin sequences, followed by a short 3' terminal U tract, usually two (1, 2, 3, or 4) U residues incorporated during transcription termination. These differences in length and composition of 5' and 3' transcript sequences flanking the dsRNA hairpin did not appear to adversely affect the ability of the dsRNA hairpin to effect dsRNA-mediated silencing, which suggests that, unlike synthetic dsRNA duplexes, endogenously expressed dsRNA hairpin constructs are effective despite varying in a number of respects, e.g., length of dsRNA "stem" between about 19-29 bp, length and composition of single-stranded loop, presence or absence of additional short 5' and/or 3' sequences.

A luciferase assay as taught in Example 1 of PCT/US05/29976 and in WO 04/076629, published 10-Sept-2004, "Methods and Constructs for Evaluation of RNAi Targets and Effector Molecules" indicated that all 4 promoter/shRNA cassettes were active in silencing their target sequences in a cell supplied with the vector and an assayable substrate. The IC50 value decreased substantially when increasing from a one promoter/shRNA cassette vector to the pHB4 expression vector containing 4 promoter/shRNA cassettes. The 1050 values may have also been affected by the relative potency and transcription levels of each shRNA molecule, and did not reflect a simple relationship to the concentration of the vector only, which in effect behaved as four drugs after entering the cell and expressing the four encoded dsRNA molecules. In other words, the increased potency reflected not only the greater number of total shRNA transcripts generated by the vector, but the also the individual potency that each shRNA has to effect the reduction of sAg or eAg production via degradation of the target viral RNA molecules. The progressive addition of shRNA cassettes increased the potency of the vector in an apparently

quantitative manner, and furthermore increased the pharmacological activity against the HBV target by inhibiting four distinct sites of the HBV target. It is important to recognize, however, that the ability of the multiple polymerase III expression constructs to express multiple individual antiviral dsRNA hairpin molecules is of significant value in and of itself, not just

5 because of associated increases in “potency”. Where the level of antiviral efficacy is high, the incremental quantitative increase in viral inhibition seen with each additional dsRNA molecule may be less important per se than the ability of the constructs to deliver what is in effect a multi-drug regimen, with the inherent advantage of being highly resistant to the development of viral resistance.

10 The expression vectors, designed to deliver multiple dsRNA effector molecules targeting highly conserved HBV and/or HCV polynucleotide sequences, when delivered to a virally infected cell, have the unique ability to destroy the viral nucleic acid products directly. Moreover, inherent and integral to the design and intent of these multiple promoter vectors (which express a plurality of different inhibitory short hairpin dsRNAs targeting different

15 portions of the viral genome), is the property of generating multiple different viral antagonists simultaneously. The antagonists (short hairpin dsRNA effector molecules) target different genome sequences in the viral genome. One of these antagonists would probably be sufficient to disable the virus; however, the redundancy serves as a “backup” mechanism such that if the viral sequence mutates to render one antagonist inert, there are 2, 3, 4 or more additional antagonists

20 available. Additionally, by targeting multiple sites in the viral genome, different DNA or RNA products of the virus which play different roles in the disease pathology can be attacked at the same time.

In the case of Hepatitis B for example, in one embodiment, the instant invention uses 4, 5, or more shRNA molecules selected from the following sequences and other highly conserved 25 HBV sequences as taught herein: e.g., “799” (SEQ ID No. 49); “1907” (SEQ ID No. 19); “2791” (SEQ ID No. 23); “1737” (SEQ ID No. 18), “1991” (SEQ ID No. 22), “1943” (SEQ ID No. 21). Other of the conserved HBV sequences disclosed herein, including sequences of e.g., 19 to 29 nucleotides, which comprise all or part of “799”, “1907”, “2791”, “1737”, “1991”, or “1943”, may be selected for inclusion in dsRNA hairpin effector molecules to be expressed by 30 expression vectors comprising multiple promoters, including multiple polymerase III expression vectors. Due to the nature of HBV gene expression and overlapping transcriptional products this

allows targeting of multiple RNA transcripts as well as the replicative template of the virus which will interfere with replication and expression of more than one of the viral proteins. One of the shRNA molecules, “1737” (SEQ ID No. 18) uniquely can disable the RNA encoding a product known as the X protein (HbX). Strong evidence exists in the biomedical literature that

5 the X protein plays a role in establishment and/or maintenance of liver cancer. Because the existing drugs that can to some extent inhibit viral replication cannot eliminate the cell of integrated or other residual copies or portions of the viral genome, these drugs cannot shut off the production of HbX, even in patients “cured” of infectious HBV, and thus can not directly reduce any incidence of cancer that is mediated by dormant HbX. Multiple anti-HBV dsRNA

10 hairpin expression constructs of the present invention can attack both the replication of the virus and the expression of all viral proteins, some which cause the inflammatory insult which results in hepatitis, and others such as HbX, which are believed to promote hepatocellular carcinoma via a distinct but not fully understood mechanism. It is recognized that the principles taught herein can be used to design constructs of the invention specifically tailored to treat such “post

15 infection” patients, which express dsRNAs against Hbx and any other residual HBV antigens.

While the HBV target sequences of the invention were chosen expressly to represent those highly conserved or identical among a large number of different isolates (strains) of HBV, for reference purposes the identified sequences, e.g., shRNA sequences, can be mapped back to HBV isolate AYW. It should be recognized, therefore, that the dsRNAs and dsRNA expression constructs of the invention are expected to be effective not only against HBV AYW and related viral strains, but against nearly all if not all HBV strains encountered in infected human populations in widely dispersed geographical areas.

EXAMPLE 2

25 Hepatitis C- Sequences for RNAi Therapeutic Development

Experiment 1

Brief Introduction:

The hepatitis C virus (HCV) is the primary cause of non-A, non-B transfusion-associated hepatitis and accounts for more than 200 million hepatitis cases worldwide. The HCV genome

30 has a high degree of sequence variability. There are six major genotypes comprising more than fifty subtypes and significant heterogeneity hallmark by quasi-species has been found within

patients. Great progress in understanding HCV replication has been made by using recombinant polymerases or cell-based subgenomic replicon systems. By using a replicon cell system, HCV-specific siRNA has been demonstrated to be able to suppress HCV protein expression and RNA replication. Sequences of the 5' NTR and both structural and nonstructural genes have been
5 targeted successfully. The highly conserved nature of the 3' NTR sequence makes it a highly attractive target for siRNA based therapy. However, no study has been done to examine the feasibility of using the 3' NTR. Here we report the design and testing of several siRNAs that can inhibit HCV protein expression in the subgenomic replicon system. Exogenously synthesized HCV-specific siRNAs were transfected into the HCV replicon cell line as described below.

10 **Cell culture and media:**

The HCV replicon in hepatoma Huh7 cells was cultured in Dulbecco's Modified Eagle Media ("DMEM") (Invitrogen) containing 10% fetal calf serum (Invitrogen), 1% penicillin-streptomycin, 1% non-essential amino acids and 0.5 mg/mL Geneticin. Cells were grown to 75% confluence prior to splitting.

15 **Western blot analysis:**

Total cell lysates from replicon cells were harvested from replicon cells in 1 x LDS Buffer (Invitrogen). The lysates were heated at 90 °C for 5 min in the presence of beta-mercaptoethanol before electrophoresis on a 10% Tris-Glycine polyacrylamide gel (Invitrogen). The protein was transferred to PVDF (Invitrogen) membrane. Following the transfer, the
20 membrane was rinsed once with PBS containing 0.5% Tween-20 (PBS Tween) and blocked in PBS-Tween containing 5% non-fat milk for 1 hr. After washing with PBS-Tween, the membrane was incubated with the primary α -NS5A antibody (a gift from Dr. Chen Liu) at 1:1500 dilution for 1 hr at room temperature. Prior to incubation with HRP conjugated a-mouse IgG secondary antibody (Amersham) diluted 1:5000, the blot was washed in PBS-Tween 20.
25 Following the secondary antibody incubation, the blot was washed again and treated with ECL (Amersham) according to the manufacturer's protocol.

Northern blot:

Total cellular RNA was extracted by using the Rneasy® kit (Qiagen). Northern blot analysis was done according to the protocol of Guo et al. Briefly, 5 μ g total RNA was

electrophoresed through a 1.0% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane and immobilized by UV cross-linking (Stratagene). Hybridization was carried out using α -[³²P]CTP-labeled neomycin RNA in a solution containing 50% deionized formamide, 5x SSC (750 mM sodium chloride, 750 mM sodium citrate), Denhardt's solution,

5 0.02 M sodium phosphate (pH 6.8), 0.2% sodium dodecyl sulfate ("SDS"), 100 μ g of sheared denatured salmon sperm DNA/ml, and 100 pg of yeast RNA/ml, for 16 hr at 58 °C. The membranes were washed once in 2x SSC/0.1% SDS for 30 min at room temperature and twice in 0.1x SSC/0.1% SDS for 30 min at 68 °C. Membranes were exposed to X-ray film.

Transfection of siRNA into replicon cells:

10 For transfection of siRNA into replicon cells the Lipofectamine® 2000 reagent (Invitrogen) was used according to the user manual. Briefly, 2x10⁴ cells in 0.5 mL of DMEM was seeded in 24 well plates one day before the transfection. The indicated amount of siRNA was diluted in 50 μ L OptiMEM and mixed with diluted Lipofectamine® 2000 reagent (1 μ L in 50 μ L of Optimem). The mixture was incubated at room temperature for 20 min before being 15 applied onto the cell monolayer. 48-72 hr after transfection, cells were washed in PBS and lysed in 100 μ L. SDS sample buffer.

Table 14

siRNA number	SEQ ID NO	HCV sequence
#12	28	GCTAACACCTCCAGGCCAATACCTGTCTC
#22	29	TCCTTGGTGGCTCCATCTTACCTGTCTC
#32	30	GCTCCATCTTAGCCCTAGTCACCTGTCTC
#42	31	TCTTAGCCCTAGTCACGGCTACCTGTCTC
#52	32	CCTAGTCACGGCTAGCTGTGACCTGTCTC
#62	33	CTAGTCACGGCTAGCTGTGAACCTGTCTC
#72	34	CGTGAGCCGCTTGACTGCAGACCTGTCTC
#82	35	GCTGATACTGGCCTCTGCACCTGTCTC
#102	36	ACTGGCCTCTGCAGATCAACCTGTCTC

20 Several short duplex dsRNAs comprising the HCV sequences identified above in Table 14 (in each case, the first 21 bases constitute conserved HCV sequences of the invention, followed by an 8-base "adapter" sequence, "CCTGTCTC", appended from the Ambion kit used

in synthesis, but which do not appear in the dsRNA effector molecules) targeting the 3'UTR; siRNA #12 targeting the HCV NS5B gene (positive control); the identified HCV core siRNA (positive control); and the identified lamin siRNA (negative control) were synthesized using the Silencer siRNA construction kit, Catalog # 1620 (Ambion Inc., Austin, Tex.). DNA oligonucleotides were synthesized by IDT (Coralville, Iowa).

Table 14A

siRNA number	SEQ ID NO	HCV sequence
#12	63	GCTAAACACTCCAGGCCAATA
#22	64	TCCTTGTTGGCTCCATCTTA
#32	65	GCTCCATCTTAGCCCTAGTCA
#42	66	TCTTAGCCCTAGTCACGGCTA
#52	67	CCTAGTCACGGCTAGCTGTGA
#62	58	CTAGTCACGGCTAGCTGTGAA
#72	69	CGTGAGCCGCTTGACTGCAGA
#82	70	GCTGATACTGGCCTCTTGCA
#102	71	ACTGGCCTCTTGCAAGATCAA

Several siRNAs comprising the HCV sequences identified above in Table 14 targeting the 3'UTR; siRNA #12 targeting the HCV NS5B gene (positive control); the identified HCV core siRNA (positive control); and the identified lamin siRNA (negative control) were synthesized using the Silencer siRNA construction kit, Catalog # 1620 (Ambion Inc., Austin, Tex.). DNA oligonucleotides were synthesized by IDT (Coralville, Iowa).

Control siRNAs:

- 15 1. HCV core (positive control): SEQ ID NO:45
2. #12, shown in Table 14, targeting the HCV NS5B gene, also a positive control
3. lamin sequence (negative control): SEQ ID NO:46

Three siRNAs were used as controls: siRNA targeting the cellular gene Lamin for negative control; siRNA targeting the core sequence of HCV as a positive control; siRNA targeting the HCV NS5B gene as a positive control. Two concentrations of each siRNA (9 and 20 pmole) were used and the results were compared with transfection of no siRNA. Accordingly, the Western Blots in **FIG. 13** represent 0, 9, and 20 pmoles of the identified siRNAs. siRNA #22, 32, 42, 62, and 72 were notably active in repressing HCV NS5A protein

expression. Presumably, HCV RNA level is also decreased based on the results obtained previously with positive control siRNA for core. Several siRNAs had minimum effect at the concentrations tested and should be evaluated at higher concentrations. These include #12 (targeting NS5B), #102, #52, and #82.

5 Experiment 2

Experiment 2 was performed as described in Experiment 1 of Hepatitis C-Sequences for RNAi Therapeutic Development except that siRNAs R1-R8, comprising the sequences (and their complements) set forth in Table 15 below, were used in transfections. The Western Blot assay performed here was as described in Example 2, Experiment 1. The control HCV core siRNA 10 used as a positive control is the siRNA described in the previous HCV Experiment 1. All siRNAs were transfected at concentrations of 0, 9, and 20 pmole except the control “core” siRNA, which was transfected at levels of 0, 3, and 9 pmole. R1, R2, R3, R5, R7, and R8 all exhibited significant inhibition of HCV as can be seen in the Western Blot, **FIG. 14**.

Table 15

siRNA	SEQ ID NO	HCV sequence
R1	37	CTGGCCTCTCTGCAGATCAAG
R2	38	TGCAGAGAGTGCTGATACTGG
R3	39	TGAGCCGCTTGACTGCAGAGA
R4	40	GAAAGGTCCGTGAGCCGCTT
R5	41	TAGCTGTGAAAGGTCCGTGAG
R6	42	TTAGCCCTAGTCACGGCTAGC
R7	43	TCCATCTTAGCCCTAGTCACG
R8	44	TTGGTGGCTCCATCTTAGCCC

15 All siRNAs evaluated map to the 3'UTR of the HCV genome and are conserved amongst HCV genotypes and quasi-species. SEQ ID NO:27 represents this 101 nt sequence of the HCV 3'UTR, sometimes referred to as the “X” region.

EXAMPLE 3

20 Silencing HBV replication and expression in a replication competent cell culture model

Brief description of cell culture model:

A human liver derived cell line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession #s V01460 and 5 J02203. Following internalization into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirrors HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg (Hepatitis B Surface 10 Antigen) particles into the media of transfected cells. This transfection model reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression and replication.

In this model, cells are co-transfected with the infectious molecular clone of HBV and the individual effector RNA constructs to be evaluated. The cells are then monitored for loss of 15 HBV expression and replication as described below.

The following is an example of an experiment using eiRNA vectors encoding sequences derived from SEQ ID NO:1 and SEQ ID NO:5. The particular eiRNA vectors for this experiment are T7 RNA polymerase-based (See, e.g., the teaching of WO 0063364, with respect to T7 dsRNA expression systems, as well as USSN 60/399,998P, filed 31-Jul-2002 and USSN 20 60/419,532, filed 18-Oct-2002) and encode hairpin RNA structures (especially desirable are, e.g., “forced” hairpin constructs, partial hairpins capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002 and PCT/US2003/024028, filed 31-Jul-2003, as well as the “udderly” structured hairpins (e.g., multi-hairpin long dsRNA vectors and multi-short hairpin structures), hairpins with mismatched 25 regions, and multiepitope constructs as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, filed 20-Oct-2003). It is expected that similar results will be obtained using other expression and promoter systems, e.g., as described above, and/or vectors encoding alternative dsRNA structures (i.e. duplex).

Experimental Procedure: Transfection.

Huh7 cells are seeded into six-well plates such that they are between 80-90% confluence at the time of transfection. All transfections are performed using LipofectamineTM (Invitrogen) according to the manufacturer's directions. In this experiment, cells are transfected with 50 ng of the infectious HBV plasmid, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid below) 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:1 (described below) and 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:5 (described below). Control cells are transfected with 50 ng of the HBV plasmid and 1 µg of the T7 RNA polymerase expression plasmid. An inert filler DNA, pGL3-basic (Promega, Madison WI), is added to all transfections to bring total DNA/transfection up to 2.5 µg DNA.

Monitoring cells for loss of HBV expression.

Following transfection, cells are monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion and DNA-containing viral particle secretion. Cells are monitored by assaying the media of transfected cells beginning at 2 days post dsRNA administration and every other day thereafter for a period of three weeks. The Auszyme ELISA, commercially available from Abbott Labs (Abbott Park, IL), is used to detect hepatitis B surface antigen (HBsAg). HBsAg is measured since HBsAg is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface antigen cistron in the transfected infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only viral replication but also replication-independent synthesis of HBsAg. Secretion of virion particles containing encapsidated HBV genomic DNA is also measured. Loss of virion particles containing encapsidated DNA is indicative of a loss of HBV replication. Analysis of virion secretion involves a technique that discriminates between naked, immature core particles and enveloped infectious HBV virions [6].

Briefly, pelleted viral particles from the media of cultured cells are subjected to Proteinase K digestion to degrade the core proteins. Following inactivation of Proteinase K, the sample is incubated with RQ1 DNase (Promega, Madison, WI) to degrade the DNA liberated from core particles. The sample is digested again with Proteinase K in the presence of SDS to inactivate the DNase as well as to disrupt and degrade the infectious enveloped virion particle. DNA is

then purified by phenol/chloroform extraction and ethanol precipitated. HBV specific DNA is detected by gel electrophoresis followed by Southern Blot analysis.

Results will desirably indicate a 70-95% decrease in both HBsAg and viral particle secretion in the media of cells transfected with the HBV plasmid, T7 RNA polymerase

- 5 expression plasmid and eiRNA constructs relative to cells transfected with only the HBV plasmid and T7 RNA polymerase expression plasmid.

Vectors used in experiment

Sequence of the T7 RNA polymerase gene

- SEQ ID NO:47 represents the T7 RNA polymerase gene which is cloned into a
10 mammalian expression vector such as pCEP4 (Invitrogen, Carlsbad, CA). Cloning can be easily done by one skilled in the art. One skilled in the art would also be aware that a leader sequence with a Kozak sequence needs to be cloned in directly upstream from the T7 RNA polymerase gene.

eiRNA vector encoding RNA hairpin derived from SEQ ID NO:1

- 15 The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 3004-2950 (about 55 bp) of GenBank accession #s V01460 and J02203. One region of the hairpin encodes the sense version of the sequences and the second region of the hairpin encodes the antisense version of this sequence. Hairpins can easily be designed and made by those skilled in the art.

20 eiRNA vector encoding RNA hairpin derived from SEQ ID NO:5

The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 730-786 of GenBank accession #s V01460 and J02203. The hairpin is designed as described for hairpin encoding sequences from SEQ ID NO:1.

25 EXPERIMENT 1

Rationale for mouse models:

Chimpanzees represent the only animal model in which to study human HBV infectivity. Mouse models are available, however, in which human HBV expression and replication occur. These models have been invaluable for the evaluation of anti-HBV therapeutic agents and have

been shown to be a predictor for the efficacy of these agents in humans [4]. The first of these models are transgenic mouse models, in which the HBV genome or selected HBV genes are expressed [7,8]. Because HBV is integrated into the mouse genome, these animals serve as a model not only for viral replication but also for RT-independent expression of antigen. A similar model exists in which replication competent HBV is expressed transiently from episomal HBV DNA. This model is created by introducing replication competent HBV DNA into mouse liver by hydrodynamic delivery [1]. Unlike the transgenic animals, these mice are not immunotolerant to HBV antigens and immune-mediated clearance of HBV transfected hepatocytes can be studied.

Although woodchuck and duck models exist for the study of woodchuck hepatitis (WHBV) and duck hepatitis (DHBV) respectively, we have opted not to use these models for several reasons. 1) Human HBV cannot be studied in these models. As we are ultimately interested in down-regulating expression of human HBV, use of these models would at some point necessitate the re-design and evaluation of vectors and/or RNAs specific for human HBV. 2) the mice are isogenic and therefore noise due to genetic variables within the system does not arise. 3) Unlike human HBV, there are no validated WHBV/DHBV cell culture models that can be studied in parallel with their respective animal models.

The experiment described below utilizes hydrodynamic delivery as a method to co-deliver replication competent HBVayw plasmid with the various effector dsRNA (eiRNA) expression vectors. Hydrodynamic delivery is ideal for this experiment because it results in efficient delivery of nucleic acid to the liver [5]. Combination of the dsRNA effector plasmid and replication competent HBV plasmid into the same formulation increases the likelihood that both plasmids are taken up by the same cells. Because expressed effector dsRNA are present in the majority of cells bearing the replicating HBV plasmid, observed results can be attributed to the performance of the effector plasmid rather than to differences in delivery efficiencies. This experiment demonstrates only that a particular eiRNA is efficacious in an infected liver. Formulation and delivery are not addressed by this example. Formulation, dosing and delivery of the eiRNA vector are enabled in the example in which transgenic mice are used.

Experimental procedure:

Control B10.D2 mice are hydrodynamically injected with an infectious molecular clone of HBV (ayw subtype) consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1,2,3]. Following internalization into hepatocytes and nuclear localization, transcription of HBVayw plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication [1]. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the sera of injected animals.

Animals are injected with four doses of the HBV replicon plasmid (1 µg, 3 µg, 5 µg, and 10 µg).

These doses are chosen because they represent non-saturating doses capable of eliciting detectable expression of a reporter plasmid following hydrodynamic delivery. Animals are co-injected with the effector dsRNA expression vector (eiRNA) such that animals in each group receive a 10-19 µg dose of a particular effector construct(s) such that the total DNA dose is 20 µg. For example in mice receiving the 3 µg dose of the HBV replicon, 17 µg of the chosen eiRNA vector(s) is injected for a total of 20 µg injected DNA. The amount of this dose is therefore dependent upon the dose of HBV plasmid used. Control animals are injected with the HBV replicon but not with an eiRNA vector. Control mice are instead co-injected with an inert filler DNA, pGL3-basic (Promega, Madison, WI) such that the total amount of DNA in the formulation is 20 µg. eiRNA vectors in this study are the U6-based expression plasmids, e.g., Ambion, Inc., Austin, TX, USA. These vectors encode short hairpin RNAs derived from SEQ ID NO:1 and SEQ ID NO:4. The exact sequences encoded by these vectors are described below. The vectors are co-injected in equal amounts (by weight). It is expected that similar results will be obtained using other expression and promoter systems as described elsewhere herein and/or vectors encoding alternative structures (i.e. duplex).

Description of U6-based eiRNA vector encoding sequences derived from SEQ ID NO:1: vector encodes a hairpin containing sequences mapping to coordinates 2905-2929 of accession #s V01460 and J02203 (i.e. the hairpin contains the sense and antisense version of this sequence, separated by a loop structure of TTCAAAAGA). Description of U6-based vector sequences can be found in Lee et al. [9]. The second eiRNA vector used in this experiment encodes a hairpin derived from SEQ ID NO:4 and encodes sequences mapping to coordinates 1215-1239 of Accession # V01460 and J02203.

Liver samples are taken from injected animals on day 1 following injection and analyzed for the presence of HBV RNA. This time point has been selected based on published results from Dr. Chisari's laboratory which detail the kinetics of HBVayw plasmid replication in mice following hydrodynamic delivery and demonstrates that peak RNA expression occurs in the liver

5 on day 1 following hydrodynamic delivery [1]. The presence of HBV RNA in liver samples is ascertained by Northern blot analysis. Liver tissue will be evaluated for the down-regulation of HBV RNA expression. In addition, serum will be collected from day 4 mice for measurement of HBVsAg and DNA-containing viral particles. Assays will be as described for the cell culture replicon experiment (Example 3) and as in Yang et al. [1]. Each vector and control group will

10 be comprised of 2 sets of animals, each set corresponding to a collection time point. There are 5 animals in each set.

Results:

Mice that are injected with the HBV replicon and the eiRNA constructs will have

15 decreased HBV-specific RNA, and HBsAg and HBV viral particles as compared to the control animals. In individual animals, decreases will range from about 70% to near 100%.

EXPERIMENT 2

Transgenic mouse studies: Background.

We will be using the HBV transgenic mouse model developed in Dr. Chisari's laboratory [8]. These mice replicate appreciable amounts of HBV DNA and have demonstrated their utility as an antiviral screen that is a predictor of human efficacy [4]. These animals are also ideal in that they are a model for HBV-integrand-mediated expression of antigen and thus can serve as a model not only for viral replication but also for RT-independent expression of antigen. This is important as we are interested in targeting not only viral replication but integrant-mediated antigen expression as well.

These experiments differ from the hydrodynamic delivery experiments in that the effector plasmids are administered to animals using clinically relevant nucleic acid delivery methods. Effectiveness in this model demonstrates efficient delivery of the effector plasmids to mouse

30 hepatocytes.

Experiment.

Mice described in reference [8] will be injected IV with a formulation containing the eiRNA vectors described in the hydrodynamic delivery example. These are the U6-based eiRNA vectors encoding hairpins containing sequences derived from SEQ ID NO:1 and SEQ ID NO:4.

5 Formulation of DNA to be injected.

DNA is formulated with trilactosyl spermine and cholesteryl spermine as described in PCT/US03/14288, "Methods for Delivery of Nucleic Acids", Satishchandran, filed 06-May-2003. Briefly, three formulations are made, all using a charge ratio of 1.2 (positive to negative charge). However, it should noted that formulations with charge ratios between 0.8 and 1.2 are 10 all expected to exhibit efficacy. The DNA starting stock solution for each plasmid is 4 mg/ml. The two plasmid stock solutions are mixed together in equal amounts such that each plasmid is at 2 mg/ml. This plasmid mixture is used for the final formulating. Formulation is as described in PCT/US03/14288 (above): Formulation A) 35% trilactosyl spermine, 65% cholesteryl spermine, Formulation B) 50% trilactosyl spermine, 50% cholesteryl spermine and Formulation C) 80% 15 trilactosyl spermine, 20% cholesteryl spermine. All resultant formulations now contain each plasmid at 1 mg/ml.

Mice are IV injected with 100 µl formulated DNA. One group of mice receives Formulation A, a second group receives Formulation B and a third group receives Formulation C. Three groups of control mice are similarly injected with formulations containing a control 20 DNA, pGL3Basic (Promega, Madison WI), Formulations D, E and F. Injections are carried out once a day for four consecutive days. Injecting for only 1-3 days is efficacious, however, more robust efficacy is seen with a four day injection protocol.

Following administration, HBV RNA and serum levels of HBsAg and DNA containing viral particles will be quantitated on days 5 and 9 post first injection. All analyses will be as 25 described for the hydrodynamic delivery studies.

Results:

HBV-specific RNA levels, HBsAg and virus containing DNA particles will have decreased relative to controls in the Formulation A, B and C groups.

30

EXAMPLE #4

**Silencing HBV replication and expression
in a replication competent cell culture model**

Brief description of cell culture model:

A human liver derived cell line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession AF090840. Following internalization into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the media of transfected cells. This transfection model, reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression and replication.

In this model, cells were co-transfected with the infectious molecular clone of HBV and an eiRNA construct. The cells were then monitored for loss of HBV expression and replication as described below.

The following is an example of an experiment that was performed using an eiRNA vector encoding sequences derived from both SEQ ID NO:1 and SEQ ID NO:2. The particular eiRNA vector used for this experiment is T7 RNA polymerase-based and encodes a duplex RNA of about 650 bp (See e.g., WO 00/63364, filed April 19, 2000). It is expected that similar results would be obtained using other expression and promoter systems as described elsewhere herein and/or vectors encoding alternative structures (i.e. duplex).

25

Experimental Procedure: Transfection.

Huh7 cells were seeded into six-well plates such that they were between 80-90% confluence at the time of transfection. All transfections were performed using Lipofectamine™ (InVitrogen) according to the manufacturer's directions. In this experiment, cells were transfected with A) 50 ng of the infectious HBV plasmid adw subtype, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid in Example 3), and 1.5 µg of the HBV-

specific eiRNA vector (described below); B) 50 ng of the infectious HBV plasmid, 1 µg of the T7 RNA polymerase expression plasmid and 1.5 µg of an irrelevant dsRNA expression vector; C) 125 ng of the infectious HBV plasmid, 1 µg of the T7 RNA polymerase expression plasmid and 1.4 µg of the HBV-specific eiRNA vector; and D) 125 ng of the infectious HBV plasmid,

- 5 1 µg of the T7 RNA polymerase expression plasmid and 1.4 µg of an irrelevant dsRNA expression vector. All transfections were carried out in duplicate. In this experiment transfections B and D served as controls. Four days post-transfection, media was removed from transfected cells and assayed for the presence of HBsAg (see below). Media from untransfected cells was also assayed as a background control.

10

Monitoring cells for loss of HBV expression.

Following transfection, cells were monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion. Cells were monitored by assaying the media of transfected cells (and a media control) at 4 days post-dsRNA administration. The Auszyme

- 15 ELISA, commercially available from Abbott Labs (Abbott Park, IL), was used to detect hepatitis B surface antigen (HBsAg). HBsAg was measured since it is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only viral replication but also replication-
- 20 independent synthesis of HB sAg.

Results:

Cells transfected with the HBV-specific eiRNA construct exhibited an 82-93% decrease in HBsAg at the four-day timepoint relative to the control transfections.

25

HBV-specific eiRNA used in this experiment

The eiRNA vector encodes a dsRNA mapping to coordinates 2027–2674 of Gen Bank Accession # AF090840. The sequence therefore includes sequences derived from both SEQ ID NO:1 and SEQ ID NO:2. More specifically, the sequence includes all of SEQ ID NO:2 and 134
30 by derived from SEQ ID NO:1.

EXAMPLE #5The down-regulation of HCV in a cell culture replicon modelBrief description

In this experiment, a cell line is created which expresses functional HCV replicons.

- 5 Creation of the cell line is as detailed in Lohmann et al. [10]. In this experiment Huh7 cells are used as the parental cell line but in theory any human hepatocyte derived cell line can be used. The cells are then transfected with an HCV specific eiRNA vector. The presence of HCV-specific RNA is ascertained by Northern blot analysis as described in Lohmann et al. [10] at days 3-7 post-transfection of eiRNA.

10

Experimental protocol: Transfection.

- Huh7 cells expressing HCV replicons are seeded into six-well plates such that they are between 80-90% confluence at the time of transfection. All transfections are performed using LipofectamineTM (InVitrogen) according to the manufacturer's directions. In this experiment, 15 cells are transfected with 1 µg of a T7 RNA polymerase expression plasmid (plasmid described in Example 3) and 1.5 µg of a T7-based eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:11 (vector described at end of example). Control cells are transfected with 1 µg T7 RNA polymerase expression plasmid and 1.5 µg of the HBV-specific (SEQ ID NO:1 specific) T7-based eiRNA vector described in Example 3. Untransfected 20 replicon-expressing HuH 7 cells are included as a second control. Each transfection mix is made such that ten transfections can be performed/mix resulting in a total of 20 transfections (10 per mix). At days 3, 4, 5, 6, and 7, two wells of cells/each transfection are lysed and RNA is extracted using standard techniques. Samples are analyzed simultaneously by Northern blot analysis for the presence of HCV-specific RNA as described in Lohmann et al. [10].

25

Results

Cells transfected with the HCV-specific eiRNA vector will show decreased HCV-specific RNA levels relative to the control cells at every time-point analyzed.

- 30 HCV-specific eiRNA vector.

The eiRNA vector is T7-based and encodes a hairpin RNA. One side of the hairpin comprises SEQ ID NO:48.

This sequence is followed by a loop structure of 9 Ts. The second side of the hairpin contains a sequence that is complementary to the first side of the hairpin. One skilled in the art
5 can easily design and construct hairpin constructs. Note: it is anticipated that other types of eiRNA vectors driven by other promoters and encoding other types of RNA structures will have similar effects.

EXAMPLE #6

10 Treatment of an HBV/HCV co-infection

Brief description

In this example, cells that are replicating both HBV and HCV replicons are transfected with an eiRNA vector that encodes both HBV and HCV-specific eiRNA.

15 **Experimental protocol:**

Creation of cell lines that contain both HBV and HCV replicons.

HuH 7 cells are first engineered to express functional HCV replicons. Creation of the cell line is as detailed in Lohmann et al. [10]. After cell line establishment, the cells are transfected with an infectious HBV replicon plasmid as described in Example 3 and below in the
20 “Transfection of cells” section. In this example, the replicon is derived from the virus sequence found in Gen Bank Accession #s V01460 and J02203. Theoretically, it is also possible to first create a cell line that stably expresses the HBV replicon and then use this cell line to create one that also expresses HCV replicons. It is also possible to transfect the cells simultaneously with both the HBV and HCV replicons and select and expand cells that are replicating both HBV and
25 HCV replicons.

Transfection of cells.

In this example, the HBV and HCV eiRNAs are encoded by separate cistrons within the same vector. However, similar results are expected if the eiRNAs are encoded within the same
30 cistron or provided by separate vectors. In this example, transcription from each cistron is driven by the T7 RNA polymerase promoter and T7 RNA polymerase. Each promoter is followed by a

hairpin eiRNA which in turn is followed by a T7 terminator (FIG. 1). The cistrons in this example are converging but one could also use diverging cistrons. It should also be noted that one could use other expression systems (including viral) to produce these RNAs and one could also use other promoters, e.g., as described elsewhere herein, to drive expression of these RNAs
5 without significantly affecting efficacy. Selection of the appropriate expression systems and promoters is within the skill in this art. Also one could express other eiRNA structures, e.g., as described elsewhere herein, as well as others, described in the literature in this area. In this example, the HBV eiRNA vector encodes sequences derived from SEQ ID NO:1 and the HCV eiRNA vector encodes sequences derived from SEQ ID NO:11. Description of vector inserts is
10 located at the end of this example.

Huh7 cells are seeded into six-well plates such that they are between 80-90% confluence at the time of transfection. All transfections are performed using LipofectamineTM (Invitrogen) according to the manufacturer's directions. In this experiment, cells are transfected with 50 ng of the infectious HBV plasmid, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid is in Example 3), 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:1 (described below and in Example 3), and 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:11 (described below). Control cells are transfected with 50 ng of the HBV plasmid and 1 µg of the T7 RNA polymerase expression plasmid. An inert filler DNA, pGL3-basic (Promega, Madison WI), is added to all transfections where needed to bring total DNA/transfection up to 2.5 µg DNA. Each transfection mix is made such that ten transfections can be performed/mix resulting in a total of 20 transfections (10 per mix).

Analyses.

25 Following transfection, cells are monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion and DNA-containing viral particle secretion. Cells are monitored by assaying the media of transfected cells beginning at 2 days post dsRNA administration and every other day thereafter for a period of three weeks. The Auszyme ELISA, commercially available from Abbott Labs (Abbott Park, IL), is used to detect hepatitis B surface
30 antigen (HBsAg). HBsAg is measured since it is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected

infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only viral replication but also replication-independent synthesis of HBsAg. Secretion of virion particles containing encapsidated HBV genomic DNA is also measured. Loss of virion particles containing encapsidated DNA is indicative of a loss of 5 HBV replication. Analysis of virion secretion involves a technique that discriminates between naked, immature core particles and enveloped infectious HBV virions [6]. Briefly, pelleted viral particles from the media of cultured cells are subjected to Proteinase K digestion to degrade the core proteins. Following inactivation of Proteinase K, the sample is incubated with RQ1 DNase (Promega, Madison, WI) to degrade the DNA liberated from core particles. The sample is 10 digested again with Proteinase K in the presence of SDS to inactivate the DNase as well as to disrupt and degrade the infectious enveloped virion particle. DNA is then purified by phenol/chloroform extraction and precipitated. HBV specific DNA is detected by gel electrophoresis followed by Southern Blot analysis.

At days 3, 4, 5, 6 and 7, two wells of cells/each transfection (experimental and control) 15 are lysed and RNA is extracted using standard techniques. Samples are also analyzed by Northern blot analysis for the presence of HCV-specific RNA as described in Lohmann et al. [10].

Results.

20 Cells transfected with the HBV-HCV-specific eiRNA vector will show decreased HCV-specific RNA levels relative to the control cells at every time-point analyzed. In addition, the levels of HBsAg and HBV viral particles will also decrease relative to the control transfections.

HCV-specific eiRNA sequence.

25 The eiRNA vector is T7-based and encodes a hairpin RNA. One side of the hairpin comprises SEQ ID NO:48.

This sequence is followed by a loop structure of 9 Ts. The second side of the hairpin contains a sequence that is complementary to the first side of the hairpin. One skilled in the art can easily design and construct hairpin constructs. Note: it is anticipated that other types of 30 eiRNA vectors driven by other promoters, including RNA polymerase III promoters, and encoding other types of RNA structures, including various hairpin structures will have similar

effects. Especially desirable are, e.g., “forced” hairpin constructs, partial hairpins capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002 and PCT/US2003/024028, filed 31-Jul-2003, as well as the “udderly” structured hairpins (e.g., multi-hairpin long dsRNA vectors and multi-short hairpin structures), hairpins with mismatched regions, and multiepitope constructs as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, filed 20-Oct-2003, as well as a variety of other dsRNA structures known to those of skill in the art.

HBV-specific eiRNA-SEQ ID NO:1

10 The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 3004-2950 (About 55 bp) of GenBank accession #s V01460 and J02203. One region of the hairpin encodes the sense version of the sequences and the second region of the hairpin encodes the antisense version of this sequence. Hairpins can easily be designed and made by those skilled in the art.

15

EXAMPLE #7

Silencing HBV replication and expression in a replication competent cell culture model (see Example 1) using combinations of HBV- specific eiRNAs in multiple promoter vectors

20 As disclosed in PCT/US05/29976, filed 23-August-2005 and in U.S. Provisional Applications entitled “Multiple RNA Pol III Promoter Expression Constructs” (Serial No. 60/603622, filed 8/23/04, and Serial No. 60/629942, filed 11/22/04) the teaching of which is hereby incorporated by reference, two or more (preferably 3, 4, 5, 6 or more) of the shRNA sequences shown in Table I and SEQ ID NO:49 may be encoded in the same plasmid vector in 25 separate cistrons under the control of separate promoters for each shRNA. SEQ ID NO:49 is:

GCCTCGCAGACGAAGGTCTCAAGAGAACTTGAGACCTTCGTCTGCGAGGC

30 SEQ ID NO:49 represents the coding strand of a DNA sequence which encodes an shRNA molecule that targets an HBV conserved region. The first 21 bases of the sequence above are identical to the sense sequence of HBV mRNA from position 799 to 779 in the HBV genome,

strain AYW (numbered according to the complement strand given in Genbank Accession No. V01460). This sequence is followed by 9 bases (i.e., AGAGAACTT)representing the loop portion of the shRNA, followed by 21 bases of the reverse complementary sequence to the first 21 bases. (It will be understood that the loop sequence serves only to join the complementary sequences which form the double-stranded “stem” and therefore considerable variation in length and nucleotide sequence is acceptable within the loop region.) In a preferred embodiment, this DNA sequence will be placed in an appropriate expression vector operably under the control of a promoter, preferably an RNA polymerase III promoter such 7SK, U6, etc. The resulting RNA transcript:

10

GCCUCGCAGACGAAGGUCUCAAGAGAACUUUGAGACCUUCGUCUGCGAGGC

will assume a hairpin or stem-loop structure having 21 basepairs in a double-stranded conformation.

15 Using methods commonly employed by one skilled in the art of molecular biology, a single vector encoding two or more, preferably three or more, more preferably four or more, five or more, or all of SEQ ID NO:49, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, and SEQ ID NO:23 is constructed. A particularly preferred embodiment for pharmaceutical applications of dsRNA-mediated silencing of the HBV target comprises a single 20 expression construct encoding under the control of separate RNA polymerase III promoters, shRNAs corresponding to at least SEQ ID NO:19, SEQ ID NO:23, and SEQ ID NO:18, and optionally, SEQ ID NO:49 and/or SEQ ID NO:21. Such shRNA-expression vectors may advantageously utilize one or more RNA polymerase III promoters, including U6, 7SK, and H1 promoters in several alternative orientations and combinations. Particularly preferred constructs 25 will utilize one or more of the 7SK promoters as taught in U.S. Provisional Serial Nos. 60/603622 and 60/629942. The instant example is thus analogous to Experiment 1 in Example 1 except that instead of introducing one vector with one shRNA at a time, the applicants deliver a single plasmid construct which expresses multiple shRNAs.

30 The advantages of this approach for therapeutic applications of dsRNA silencing are principally in the economy, simplicity and coordinated delivery of a single drug entity which comprises multiple different shRNAs each targeting a different site of the HBV genome. The

ability to simultaneously target multiple sites of a viral genome is highly advantageous in preventing the clinically widespread phenomenon of drug resistance (by viral mutation), and the ability to combine dsRNA drug entities against these different target sites in a single delivery agent (the plasmid vectors of this invention) makes this conceptual approach uniquely feasible.

- 5 While shRNAs, e.g., RNAs corresponding to SEQ ID NO:49, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, and SEQ ID NO:23 may be produced, e.g., through chemical synthesis or *in vitro* expression, and delivered into an animal cell singly and in combination, there are significant advantages in some applications to express within the animal cell multiple shRNAs from a single expression vector. In this example, the potency of the multiple shRNA
10 expression vectors significantly exceeded that of any one of the single vectors used in Experiment 1, as measured by similar assays.

EXAMPLE #8

Inhibition of Infectious Virions of HCV by dsRNA Effector Molecules

- 15 As a further example of HCV-targeted dsRNAs, the sequences given in Table 16 represent highly conserved coding region sequitopes from the 5' and 3' untranslated regions of HCV. Each sequence is written as the coding strand and is used to specify the design of a short hairpin dsRNA effector molecule comprising the coding sequence as shown in Table 16 connected to its reverse complement by a loop or linker sequence as described elsewhere herein.
20 The sequences shown are predicted to be particularly efficacious as antiviral therapeutic agents because they were tested in a newly available *in vitro* HCV replication system capable of producing whole, infectious virions (disclosed in Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ., “Production of infectious hepatitis C virus in tissue culture from a cloned viral
25 genome”, Nat Med 2005 Jul;11 (7):791-6; and in Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV., “Robust hepatitis C virus infection in vitro”, Proc Natl Acad Sci USA 2005 Jun 28;102(26):9294-9). In this system, all viral proteins and viral nucleic acid sequences are present in a cell, as in a natural infection. In less complete replicon systems, dsRNA silencing molecules cannot be as rigorously tested as in
30 the new system. It is expected that one or more (2, 3, 4, 5, or more) of the HCV sequitopes and their complements could be utilized as duplex dsRNA effector molecules, short hairpin dsRNA

effector molecules, and/or encoded into dsRNA expression vectors capable of expression in vivo in a mammalian cell, including a human cell or organism.

Table 16

Seq Name	SEQ ID NO	Sequence (5' to 3')
HCV5M-5.1	72	AAAGGCCTTGTGGTACTGCCT
HCV5M-5.3	73	TTGTGGTACTGCCTGATAGGG
HCVXM-13	74	TAGCTGTGAAAGGTCCGTGAG
HCVXM-34	75	ATCTTAGCCTAGTCACGGCTAGCTG
HCVXM-35	76	TAGTCACGGCTAGCTGTGAAAGGTCCG

5 The sequences in Table 17 represent additional preferred highly conserved at least 19 contiguous base pair HCV sequences from the 5' UTR of the virus (SEQ ID NO: 11). To generate the dsRNA effector molecules of the invention, these sequences are used in conjunction with their reverse complement and, optionally, a loop or linker sequence joining the sequence to its reverse
10 complement, when it is desired to form a hairpin dsRNA effector molecule. One or more double-stranded RNA molecules comprising said conserved 5' UTR sequences (from SEQ ID NO: 11) may advantageously be used in combination with one or more other dsRNA effector molecules of the invention, including e.g., one or more highly conserved sequences from the 3' UTR (SEQ ID NO:27) and/or one or more at least 19 contiguous base pair sequences from SEQ
15 ID NO. 12.

Table 17

HCV 5' UTR siRNAs		
Sequence Name	Sequence (5' to 3')	SEQ ID NO
HCV5P-1.1	CCTGTGAGGAACACTACTGTCTT	77
HCV5P-1.2	ACGCAGAAAGCGTAGCCAT	78
HCV5P-1.3	CGTCTAGCCATGGCGTTAGTA	79
HCV5P-1.4	GTCTAGCCATGGCGTTAGTAT	80
HCV5P-1.5	CTCCCCCTGTGAGGAACACTACTGTCTT	81
HCV5P-1.6	GAGGAACACTGTCTTCACGCAGAA	82
HCV5P-1.7	GTGAGGAACACTGTCTTCACGCAGAA	83
HCV5P-2.1	GAGCCATAGTGGTCTGCCGAA	84
HCV5P-2.2	GAACCGGTGAGTACACCGGAA	85
HCV5P-2.3	ACCGGTGAGTACACCGGAA	86
HCV5P-2.4	GGGAGAGCCATAGTGGTCTGCCGAA	87
HCV5P-5.1	GGCCTTGTGGTACTGCCTGAT	88
HCV5P-5.2	GCCTTGTGGTACTGCCTGATA	89
HCV5P-5.3	GTACTGCCTGATAGGGTGCTT	90
HCV5P-5.4	AAGGCCTTGTGGTACTGCCTGATAGGG	91
HCV5P-5.5	CGAAAGGCCTTGTGGTACTGCCTGATA	92
HCV5P-5.6	CTTGCAGTGCCCCGGGAGGTCTCGTA	93
HCV5M-1.1	ATCACTCCCCTGTGAGGAAC	94
HCV5M-1.2	TTCACGCAGAAAGCGTCTAGC	95
HCV5M-1.3	TAGCCATGGCGTTAGTATGAG	96
HCV5M-1.4	ATCACTCCCCTGTGAGGAACACTG	97
HCV5M-1.5	ATCACTCCCCTGTGAGGAACACTGT	98
HCV5M-1.6	AACTACTGTCTTCACGCAGAAAGCG	99
HCV5M-1.7	AACTACTGTCTTCACGCAGAAAGCGTC	100
HCV5M-2.1	ATAGTGGTCTGCCGAAACCGGT	101
HCV5M-2.2	TAGTGGTCTGCCGAAACCGGT	102
HCV5M-2.3	AACCGGTGAGTACACCGGAATTGCC	103
HCV5M-5.2	AAGGCCTTGTGGTACTGCCTG	104
HCV5M-5.4	TACTGCCTGATAGGGTGCTTG	105
HCV5M-5.5	TTGTGGTACTGCCTGATAGGGTGCTTG	106
HCV5M-5.6	TACTGCCTGATAGGGTGCTGCGAG	107
HCV5M-5.7	TAGGGTGCTGCGAGTGCCCGGG	108
HCV5M-5.8	TTGCGAGTGCCCCGGGAGGTCTCGTAG	109

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FIG. 15

Position	sequence	length
9510-9531	TGGCTCCATCTTAGCCCTAGTC	22
9510-9533	TGGCTCCATCTTAGCCCTAGTCAC	24
9510-9534	TGGCTCCATCTTAGCCCTAGTCACG	25
9510-9535	TGGCTCCATCTTAGCCCTAGTCACGG	26
9510-9536	TGGCTCCATCTTAGCCCTAGTCACGGC	27
9514-9534	TCCATCTTAGCCCTAGTCACG	21
9514-9535	TCCATCTTAGCCCTAGTCACGG	22
9514-9536	TCCATCTTAGCCCTAGTCACGGC	23
9514-9539	TCCATCTTAGCCCTAGTCACGGCTAG	26
9514-9540	TCCATCTTAGCCCTAGTCACGGCTAGC	27
9514-9542	TCCATCTTAGCCCTAGTCACGGCTAGCTG	29
9517-9539	ATCTTAGCCCTAGTCACGGCTAG	23
9517-9540	ATCTTAGCCCTAGTCACGGCTAGC	24
9517-9542	ATCTTAGCCCTAGTCACGGCTAGCTG	26
9517-9544	ATCTTAGCCCTAGTCACGGCTAGCTGTG	28
9518-9539	TCTTAGCCCTAGTCACGGCTAG	22
9518-9540	TCTTAGCCCTAGTCACGGCTAGC	23
9518-9542	TCTTAGCCCTAGTCACGGCTAGCTG	25
9518-9544	TCTTAGCCCTAGTCACGGCTAGCTGTG	27
9520-9540	TTAGCCCTAGTCACGGCTAGC	21
9520-9542	TTAGCCCTAGTCACGGCTAGCTG	23
9520-9544	TTAGCCCTAGTCACGGCTAGCTGTG	25
9520-9548	TTAGCCCTAGTCACGGCTAGCTGTGAAAG	29
9521-9542	TAGCCCTAGTCACGGCTAGCTG	22
9521-9544	TAGCCCTAGTCACGGCTAGCTGTG	24
9521-9548	TAGCCCTAGTCACGGCTAGCTGTGAAAG	28
9521-9549	TAGCCCTAGTCACGGCTAGCTGTGAAAGG	29
9522-9542	AGCCCTAGTCACGGCTAGCTG	21
9522-9544	AGCCCTAGTCACGGCTAGCTGTG	23
9522-9548	AGCCCTAGTCACGGCTAGCTGTGAAAG	27
9522-9549	AGCCCTAGTCACGGCTAGCTGTGAAAGG	28
9527-9548	TAGTCACGGCTAGCTGTGAAAG	22
9527-9549	TAGTCACGGCTAGCTGTGAAAGG	23
9527-9551	TAGTCACGGCTAGCTGTGAAAGGTC	25
9527-9552	TAGTCACGGCTAGCTGTGAAAGGTCC	26
9527-9553	TAGTCACGGCTAGCTGTGAAAGGTCCG	27
9527-9555	TAGTCACGGCTAGCTGTGAAAGGTCCGTG	29
9528-9548	AGTCACGGCTAGCTGTGAAAG	21
9528-9549	AGTCACGGCTAGCTGTGAAAGG	22
9528-9551	AGTCACGGCTAGCTGTGAAAGGTTC	24
9528-9552	AGTCACGGCTAGCTGTGAAAGGTCC	25
9528-9553	AGTCACGGCTAGCTGTGAAAGGTCCG	26
9528-9555	AGTCACGGCTAGCTGTGAAAGGTCCGTG	28
9530-9551	TCACGGCTAGCTGTGAAAGGTTC	22
9530-9552	TCACGGCTAGCTGTGAAAGGTCC	23
9530-9553	TCACGGCTAGCTGTGAAAGGTCCG	24
9530-9555	TCACGGCTAGCTGTGAAAGGTCCGTG	26
9530-9557	TCACGGCTAGCTGTGAAAGGTCCGTGAG	28
9530-9558	TCACGGCTAGCTGTGAAAGGTCCGTGAGC	29
9532-9552	ACGGCTAGCTGTGAAAGGTCC	21
9532-9553	ACGGCTAGCTGTGAAAGGTCCG	22
9532-9555	ACGGCTAGCTGTGAAAGGTCCGTG	24
9532-9557	ACGGCTAGCTGTGAAAGGTCCGTGAG	26
9532-9558	ACGGCTAGCTGTGAAAGGTCCGTGAGC	27
9532-9559	ACGGCTAGCTGTGAAAGGTCCGTGAGCC	28
9532-9560	ACGGCTAGCTGTGAAAGGTCCGTGAGCCG	29
9537-9557	TAGCTGTGAAAGGTCCGTGAG	21
9537-9558	TAGCTGTGAAAGGTCCGTGAGC	22
9537-9559	TAGCTGTGAAAGGTCCGTGAGCC	23

9537-9560	TAGCTGTGAAAGGTCCGTGAGCCG	24
9537-9561	TAGCTGTGAAAGGTCCGTGAGCCGC	25
9537-9564	TAGCTGTGAAAGGTCCGTGAGCCGCTTG	28
9538-9558	AGCTGTGAAAGGTCCGTGAGC	21
9538-9559	AGCTGTGAAAGGTCCGTGAGCC	22
9538-9560	AGCTGTGAAAGGTCCGTGAGCCG	23
9538-9561	AGCTGTGAAAGGTCCGTGAGCCGC	24
9538-9564	AGCTGTGAAAGGTCCGTGAGCCGCTTG	27
9538-9566	AGCTGTGAAAGGTCCGTGAGCCGCTTGAC	29
9541-9561	TGTGAAAGGTCCGTGAGCCGC	21
9541-9564	TGTGAAAGGTCCGTGAGCCGCTTG	24
9541-9566	TGTGAAAGGTCCGTGAGCCGCTTGAC	26
9541-9568	TGTGAAAGGTCCGTGAGCCGCTTGACTG	28
9541-9569	TGTGAAAGGTCCGTGAGCCGCTTGACTGC	29
9543-9564	TGAAAGGTCCGTGAGCCGCTTG	22
9543-9566	TGAAAGGTCCGTGAGCCGCTTGAC	24
9543-9568	TGAAAGGTCCGTGAGCCGCTTGACTG	26
9543-9569	TGAAAGGTCCGTGAGCCGCTTGACTGC	27
9543-9571	TGAAAGGTCCGTGAGCCGCTTGACTGCAG	29
9545-9566	AAAGGTCCGTGAGCCGCTTGAC	22
9545-9568	AAAGGTCCGTGAGCCGCTTGACTG	24
9545-9569	AAAGGTCCGTGAGCCGCTTGACTGC	25
9545-9571	AAAGGTCCGTGAGCCGCTTGACTGCAG	27
9545-9573	AAAGGTCCGTGAGCCGCTTGACTGCAGAG	29
9546-9564	AAGGTCCGTGAGCCGCTTGAC	21
9546-9566	AAGGTCCGTGAGCCGCTTGACTG	23
9546-9569	AAGGTCCGTGAGCCGCTTGACTGC	24
9546-9571	AAGGTCCGTGAGCCGCTTGACTGCAG	26
9546-9573	AAGGTCCGTGAGCCGCTTGACTGCAGAG	28
9547-9568	AGGTCCGTGAGCCGCTTGACTG	22
9547-9569	AGGTCCGTGAGCCGCTTGACTGC	23
9547-9571	AGGTCCGTGAGCCGCTTGACTGCAG	25
9547-9573	AGGTCCGTGAGCCGCTTGACTGCAGAG	27
9547-9575	AGGTCCGTGAGCCGCTTGACTGCAGAGAG	29
9550-9571	TCCGTGAGCCGCTTGACTGCAG	22
9550-9573	TCCGTGAGCCGCTTGACTGCAGAG	24
9550-9575	TCCGTGAGCCGCTTGACTGCAGAGAG	26
9550-9577	TCCGTGAGCCGCTTGACTGCAGAGAGTG	28
9550-9578	TCCGTGAGCCGCTTGACTGCAGAGAGTC	29
9554-9575	TGAGCCGCTTGACTGCAGAGAG	22
9554-9577	TGAGCCGCTTGACTGCAGAGAGTG	24
9554-9578	TGAGCCGCTTGACTGCAGAGAGTG	25
9554-9580	TGAGCCGCTTGACTGCAGAGAGTGCTG	27
9556-9577	AGCCGCTTGACTGCAGAGAGTG	22
9556-9578	AGCCGCTTGACTGCAGAGAGTG	23
9556-9580	AGCCGCTTGACTGCAGAGAGTGCTG	25
9556-9584	AGCCGCTTGACTGCAGAGAGTGCTGATAC	29
9562-9584	TTGACTGCAGAGAGTGCTGATAC	23
9562-9586	TTGACTGCAGAGAGTGCTGATACTG	25
9562-9587	TTGACTGCAGAGAGTGCTGATACTGG	26
9562-9588	TTGACTGCAGAGAGTGCTGATACTGGC	27
9562-9589	TTGACTGCAGAGAGTGCTGATACTGGCC	28
9563-9584	TGACTGCAGAGAGTGCTGATAC	22
9563-9586	TGACTGCAGAGAGTGCTGATACTG	24
9563-9587	TGACTGCAGAGAGTGCTGATACTGG	25
9563-9588	TGACTGCAGAGAGTGCTGATACTGGC	26
9563-9589	TGACTGCAGAGAGTGCTGATACTGGCC	27
9563-9591	TGACTGCAGAGAGTGCTGATACTGGCCTC	29
9565-9586	ACTGCAGAGAGTGCTGATACTG	22
9565-9587	ACTGCAGAGAGTGCTGATACTGG	23

9565-9588	ACTGCAGAGAGTGCTGATACTGGC	24
9565-9589	ACTGCAGAGAGTGCTGATACTGGCC	25
9565-9591	ACTGCAGAGAGTGCTGATACTGGCCTC	27
9565-9593	ACTGCAGAGAGTGCTGATACTGGCCTCTC	29
9567-9587	TGCAGAGAGTGCTGATACTGG	21
9567-9588	TGCAGAGAGTGCTGATACTGGC	22
9567-9589	TGCAGAGAGTGCTGATACTGGCC	23
9567-9591	TGCAGAGAGTGCTGATACTGGCCTC	25
9567-9593	TGCAGAGAGTGCTGATACTGGCCTCTC	27
9567-9595	TGCAGAGAGTGCTGATACTGGCCTCTCTG	29
9570-9591	AGAGAGTGCTGATACTGGCCTC	22
9570-9593	AGAGAGTGCTGATACTGGCCTCTC	24
9570-9595	AGAGAGTGCTGATACTGGCCTCTCTG	26
9570-9596	AGAGAGTGCTGATACTGGCCTCTCTGC	27
9570-9598	AGAGAGTGCTGATACTGGCCTCTCTGCAG	29
9572-9593	AGAGTGCTGATACTGGCCTCTC	22
9572-9595	AGAGTGCTGATACTGGCCTCTCTG	24
9572-9596	AGAGTGCTGATACTGGCCTCTCTGC	25
9572-9598	AGAGTGCTGATACTGGCCTCTCTGCAG	27
9574-9595	AGTGCTGATACTGGCCTCTCTG	22
9574-9596	AGTGCTGATACTGGCCTCTCTGC	23
9574-9598	AGTGCTGATACTGGCCTCTCTGCAG	25
9574-9601	AGTGCTGATACTGGCCTCTCTGCAGATC	28
9576-9596	TGCTGATACTGGCCTCTCTGC	21
9576-9598	TGCTGATACTGGCCTCTCTGCAG	23
9576-9601	TGCTGATACTGGCCTCTCTGCAGATC	26
9576-9604	TGCTGATACTGGCCTCTCTGCAGATCAAG	29
9579-9601	TGATACTGGCCTCTCTGCAGATC	23
9579-9604	TGATACTGGCCTCTCTGCAGATCAAG	26
9581-9601	ATACTGGCCTCTCTGCAGATC	21
9581-9604	ATACTGGCCTCTCTGCAGATCAAG	24
9583-9604	ACTGGCCTCTCTGCAGATCAAG	22

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re of: Catherine J. Pachuk Confirmation No. : 3823
Serial No.: 10/560,377 Group Art Unit: 1648
Filed: June 19, 2006 Examiner: PENG, Bo
For: CONSERVED HBV AND HCV SEQUENCES USEFUL FOR GENE SILENCING

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

GENERAL TRANSMITTAL & CERTIFICATE OF EFS SUBMISSION

Pursuant to 37 C.F.R. § 1.8(a)(i)(1)(C) I hereby certify that the following documents:

1. **Renewed Petition Under §1.182 / Request to Correct Priority Under §1.78 (6 pp.);**
2. **Terminal Disclaimer Accompanying a Renewed Petition (1 pg.);**
3. **Exhibit A (94 pp.);**
4. **Exhibit B (71 pp.);**
5. **Preliminary Amendment (5 pp.);**
6. **Continuation-in-Part Specification – Marked Copy (122 pp.);**
7. **Continuation-in-Part Specification – Clean Copy (99 pp.);**
8. **Application Data Sheet (5 pp.);**
9. **Figure 15 (3 pp.); and**
10. **EFS Fee Transmittal (Petition & Disclaimer Fees - \$1950.00) (2 pp.).**

are being submitted to the Patent and Trademark Office via the Office Electronic Filing System in accordance with § 1.6(a)(4) on September 9, 2010.

AUTHORIZATION TO CHARGE/CREDIT DEPOSIT ACCOUNT

The Commissioner is hereby authorized to charge fee deficiencies or credit overpayments associated with the instant filing in above-referenced matter to NIXON PEABODY LLP Deposit Account No. 50-0850.

Respectfully submitted,

Date: September 9, 2010

/Mark J. FitzGerald/

David S. Resnick (Reg. No. 34,235)
Mark J. FitzGerald (Reg. No. 45,928)
NIXON PEABODY LLP
100 Summer Street
Boston, MA 02110-2131
Tel: (617) 345-1000
bostonpatent@nixonpeabody.com

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	051058-034000
		Application Number	
Title of Invention	CONSERVED HBV AND HCV SEQUENCES USEFUL FOR GENE SILENCING		
<p>The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.</p> <p>This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.</p>			

Secrecy Order 37 CFR 5.2

- Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

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	Catherine	J.	Pachuk	
Residence Information (Select One)		<input checked="" type="radio"/> US Residency <input type="radio"/> Non US Residency <input type="radio"/> Active US Military Service		
City	Cambridge	State/Province	MA	Country of Residence i
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Mailing Address of Applicant:				
Address 1	300 Third Street			
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Applicant 2				<input type="button" value="Remove"/>
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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	051058-034000
		Application Number	
Title of Invention	CONSERVED HBV AND HCV SEQUENCES USEFUL FOR GENE SILENCING		

Citizenship under 37 CFR 1.41(b) i	US		
Mailing Address of Applicant:			
Address 1	300 Third Street		
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City	Cambridge	State/Province	MA
Postal Code	02142	Country i	US

Applicant 4				<input type="button" value="Remove"/>
Applicant Authority		<input checked="" type="radio"/> Inventor	<input type="radio"/> Legal Representative under 35 U.S.C. 117	<input type="radio"/> Party of Interest under 35 U.S.C. 118
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	Liat		Mintz	

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Title of the Invention	CONSERVED HBV AND HCV SEQUENCES USEFUL FOR GENE SILENCING		
Attorney Docket Number	051058-034000	Small Entity Status Claimed	<input type="checkbox"/>
Application Type	Nonprovisional		
Subject Matter	Utility		
Suggested Class (if any)	Sub Class (if any)		
Suggested Technology Center (if any)			
Total Number of Drawing Sheets (if any)	16	Suggested Figure for Publication (if any)	

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	051058-034000
		Application Number	
Title of Invention	CONSERVED HBV AND HCV SEQUENCES USEFUL FOR GENE SILENCING		

Publication Information:

- Request Early Publication (Fee required at time of Request 37 CFR 1.219)

Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Enter either Customer Number or complete the Representative Name section below. If both sections are completed the Customer Number will be used for the Representative Information during processing.

Please Select One:	<input checked="" type="radio"/> Customer Number	<input type="radio"/> US Patent Practitioner	<input type="radio"/> Limited Recognition (37 CFR 11.9)
Customer Number	90162		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78(a)(2) or CFR 1.78(a)(4), and need not otherwise be made part of the specification.

Prior Application Status	Expired	<input type="button" value="Remove"/>	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
	Continuation in part of	PCT/US2004/019229	1994-06-10
Prior Application Status	Expired	<input type="button" value="Remove"/>	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
PCT/US2004/019229	non provisional of	60478076	2003-06-12
Prior Application Status	Expired	<input type="button" value="Remove"/>	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
	non provisional of	60638294	2004-12-22
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the Add button.			

Foreign Priority Information:

This section allows for the applicant to claim benefit of foreign priority and to identify any prior foreign application for which priority is not claimed. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(a).

<input type="button" value="Remove"/>			
Application Number	Country	Parent Filing Date (YYYY-MM-DD)	Priority Claimed
			<input type="radio"/> Yes <input checked="" type="radio"/> No

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	051058-034000
		Application Number	
Title of Invention	CONSERVED HBV AND HCV SEQUENCES USEFUL FOR GENE SILENCING		

Additional Foreign Priority Data may be generated within this form by selecting the button.

Assignee Information:

Providing this information in the application data sheet does not substitute for compliance with any requirement of part 3 of Title 37 of the CFR to have an assignment recorded in the Office.

Assignee 1		<input type="button" value="Remove"/>	
If the Assignee is an Organization check here. <input checked="" type="checkbox"/>			
Organization Name	ALNYLAM PHARMACEUTICALS, INC.		
Mailing Address Information:			
Address 1	300 Third Street		
Address 2	Third Floor		
City	Cambridge	State/Province	MA
Country	US	Postal Code	02142
Phone Number			Fax Number
Email Address			
Additional Assignee Data may be generated within this form by selecting the <input type="button" value="Add"/> button.			<input type="button" value="Add"/>

Signature:

A signature of the applicant or representative is required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature.

Signature	/Mark J. Fitzgerald/	Date (YYYY-MM-DD)	2010-09-09
First Name	Mark	Last Name	Fitzgerald
			Registration Number
			45928

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**